REVIEW

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Some contributions to the application of LC-NMR, LC-MS, and LC-CD to the biosynthesis of isoquinoline alkaloids using callus cultures

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Respectfully dedicated to the late Professor Dr. Dr. h.c. mult. Meinhart Zenk

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Hyphenated spectroscopic techniques in combination with a special extraction and work-up of plant calli cultures of Berberidaceae, Fumariaceae, and Papaveraceae families, e.g., enabled us to get deeper insight into the sequential biochemical conversions of precursors into simple isoquinoline- and protoberberine-alkaloids and their follow-up-products with different skeletons. Some new alkaloids of these types have been found.

1. Introduction

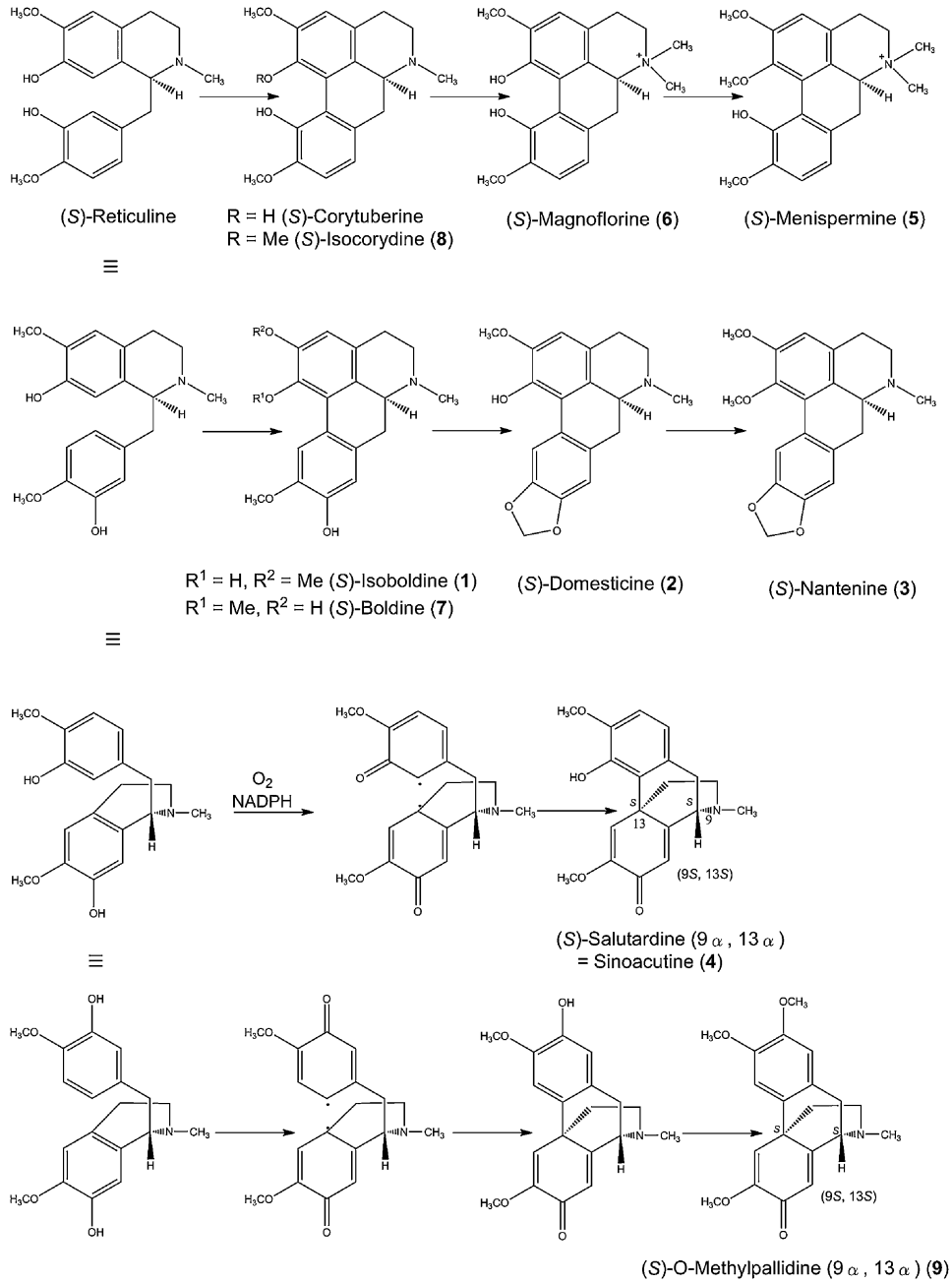
In his textbook of chemistry for pharmacists, Fischer (1904) reported that the alkaloids of opium (morphine, codeine, papaverine, laudanosine, rhoeadine, etc.) are isoquinoline-derived compounds. Nearly at the same time, Faltis (Faltis 1906; Faltis 1910) published the hypothesis that botanically related families as Papaveraceae, Fumariaceae, Menispermaceae, Berberidaceae, Annonaceae, and Ranunculaceae, e.g., should contain a common phylogenetic compound ("gemeinsame Stammsubstanz", a 1-(2′-methylcarbonyl-3′,4′-dihydroxybenzyl)-6,7-dihydroxy-tetrahydro(?) isoquinoline – no double bonds are drawn in the phenyl groups of opium- and berbine-alkaloids. Nowadays this would be termed a chemotactic marker. – This insight faded away because von Gerichten (1881) had found that zink dust destillation of morphine led to phenanthrene, which was unequivocally identified by elementary analysis and conversion to phenenthrenequinone and diphenic acid. Consequently morphine and its strictly related compounds (codeine, thebaine, etc.) were classified as phenanthrene alkaloids (Claret, e.g., 1979). – It was Awe (1934) who defined morphine as a 1-benzylisoquinoline alkaloid. The formula of morphine drawn by him shows this genetic connection. Plants of several species of the families Berberidaceae, Ranunculaceae, Menispermaceae, Papaveraceae, and Fumariaceae produce isoquinoline alkaloids with a variety of structural types. They comprise some important drugs for therapy (the analgesic morphine, the antimicrobial berberine, the antigout colchicine, the antiamoebic emetine, and the skeletal muscle relaxant tubocurarine, e.g.) and euphoria. Attempts to induce or increase the production of plant secondary metabolites (SMs) are necessary. Compared with the growth of whole plants in natural environment or on agricultural farms, growth of plant tissue cultures in bioreactors has the advantages of well controlled production without the limitations of natural factors such as geographical location and seasonal variations. We found that yields of the desired products were very low or sometimes not detectable in our dedifferentiated cells such as callus tissues or suspension cultured cells. However, exogenous supply of a biosynthetic precursor or intermediate to the culture medium increases the yield of the desired product and sometimes produces compounds not normally found in the original plant. These results show that plant cell culture systems have a potential for the production of SMs. Our efforts have focused on the development of biosynthetic activities of cultured cells by collecting knowledge of the biosynthetic pathways of SMs and by manipulating it. The way of identification of SMs is changing from structural determination after isolation into that by application of hyphenated techniques: LC-NMR, LC-MS, and LC-CD according to the development of analytic instruments.

2. Application of hyphenated techniques

LC-NMR has been used for studies of drug metabolism and identification of natural products, several aspects have been reviewed (Wolfender et al. 1998, 2001). Here we describe an extension and its application to studies of biosynthesis (Iwasa et al. 2008, 2009) exemplified by investigations of the alkaloids of the Berberidaceae species *Nandina domestica*. It contains aporphine-, proaporphine-, protoberberine- and simple isoquinoline-alkaloids, together with non-alkaloidal phenylpropane amides (Schemes 1 and 2).

Ground parts of *N. domestica* were extracted by MeOH/water, and alkaloids were separated from non-alkaloidal compounds in the extract. The alkaloid fractions so obtained were analyzed by LC-MS/MS, LC-NMR, and LC-CD. Thus, the aporphine alkaloids isoboldine **(1),** domesticine **(2),** and nantenine **(3)** besides the proaporphine alkaloid sinoacutine **(4)** were identified. Previously these four alkaloids had been isolated from *N. domestica* by Chinese, French, and Japanese groups (for references see Iwasa et al. (2008).

Apart from nantenine **(3)**, the NMR spectra (including NOE's) of these alkaloids are thoroughly discussed. This holds true also for the *N,N*-dimethyl-aporphinium-alkaloid menispermine **(5)**, the NMR-data of which were correlated with those of the related compound magnoflorine **(6).**



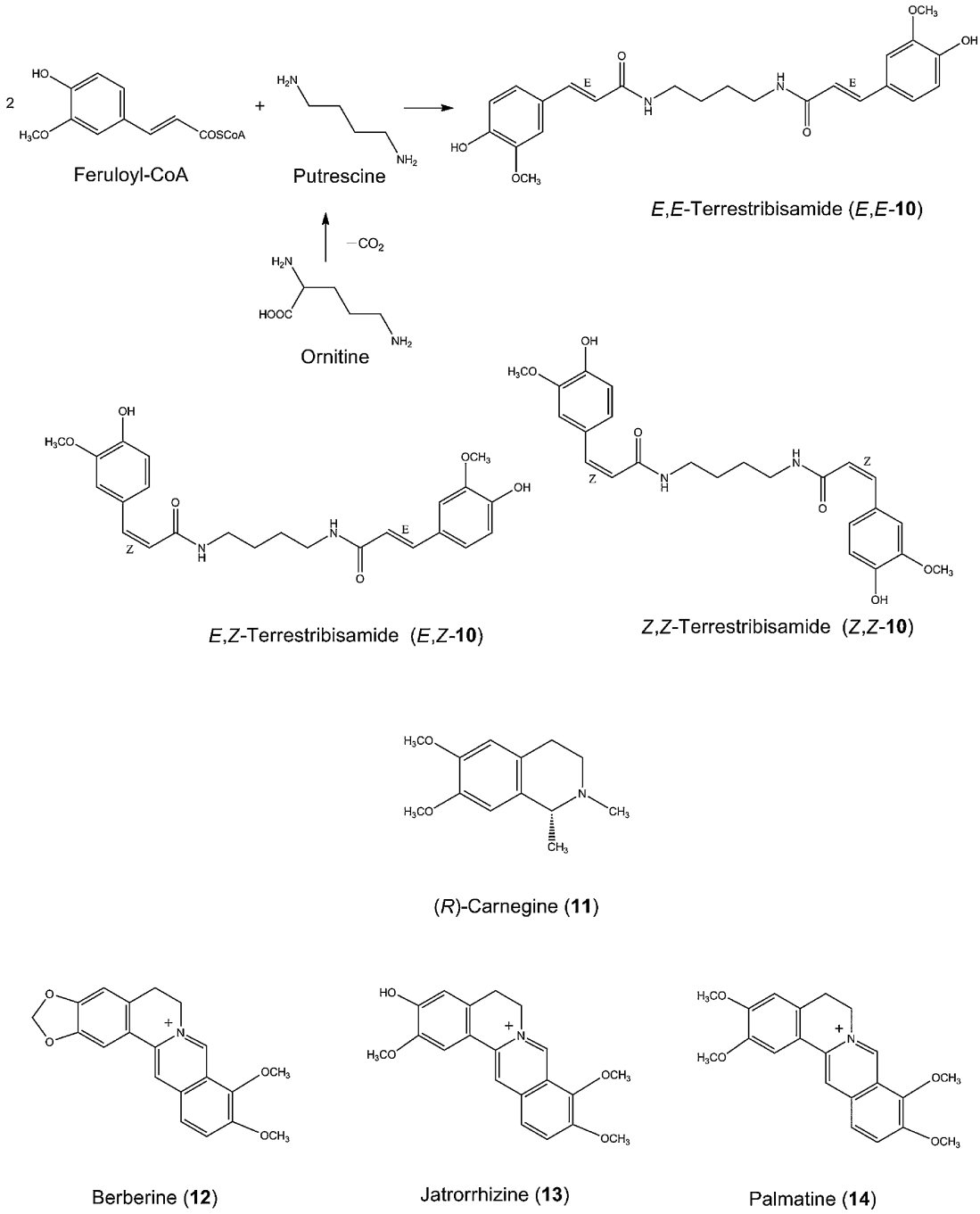
Scheme 1: Aporphines (**1**, **2**, **3**, and **5–8**) and proaporphines (**4** and **9**) and their proposed biosynthetic pathways in *Nandina domestica*: Aporphines (**6–8**) were not identified in this time

For the measurement of LC-CD spectra, the spectra of commercially available (*S*)-boldine **(7)** and (*S*)-isocorydine **(8)** were studied under stopped flow conditions, in order to find a proper wavelength. The peaks of isoboldine **(1)**, domesticine **(2)**, and nantenine **(3)** show positive CD effects at 236 nm as did the reference compounds. Thus, these *Nandina* alkaloids have *S*-configuration. A further proaporphine alkaloid is *O*-methylpallidine (**9**). This became evident by comparison of its NMR- and NOE-data to those of sinoacutine (**4**). This proaporphine alkaloid shows a negative CD effect, but an assignment of stereochemistry by determination of $[\alpha]_D$ is lacking, because a sufficient quantity of **4** could not be obtained on the conditions of our experiments.

On the other hand, it has been determined that introduction of (*S*)-reticuline (Scheme 1) in the presence of NADP-P450 reductase gave four phenol-coupled products of the *S*-series, including sinoacutine (**4**) and (+)-isoboldine (**1**) (Grobe et al. 2009). From a biosynthetic pathway it was assumed that sinoacutine (**4**) and *O*-methylpallidine (**9**) have *S*-configuration in *Nandina domestica*. For a proposal of biosynthetic pathways to aporphine- and proaporphine alkaloids in *Nandina domestica* see Scheme 1.

A non-alkaloidal compound with a characteristic 16 Hz coupling of two protons pointed towards a *trans* alkene. The NMR data are in accordance with a 3,4-dioxygenated cinnamic acid increment, and the MS revealed a dimeric structure. Summarizing all spectroscopic data, this compound is E,E-terrestribisamide **(E,E-10)**. In addition, its E,Z and Z,Z-isomers **E,Z-10** and **Z,Z-10** were identified for the first time in this plant. The terrestribisamides may result from ornithine and ferulic acid (Scheme 2).

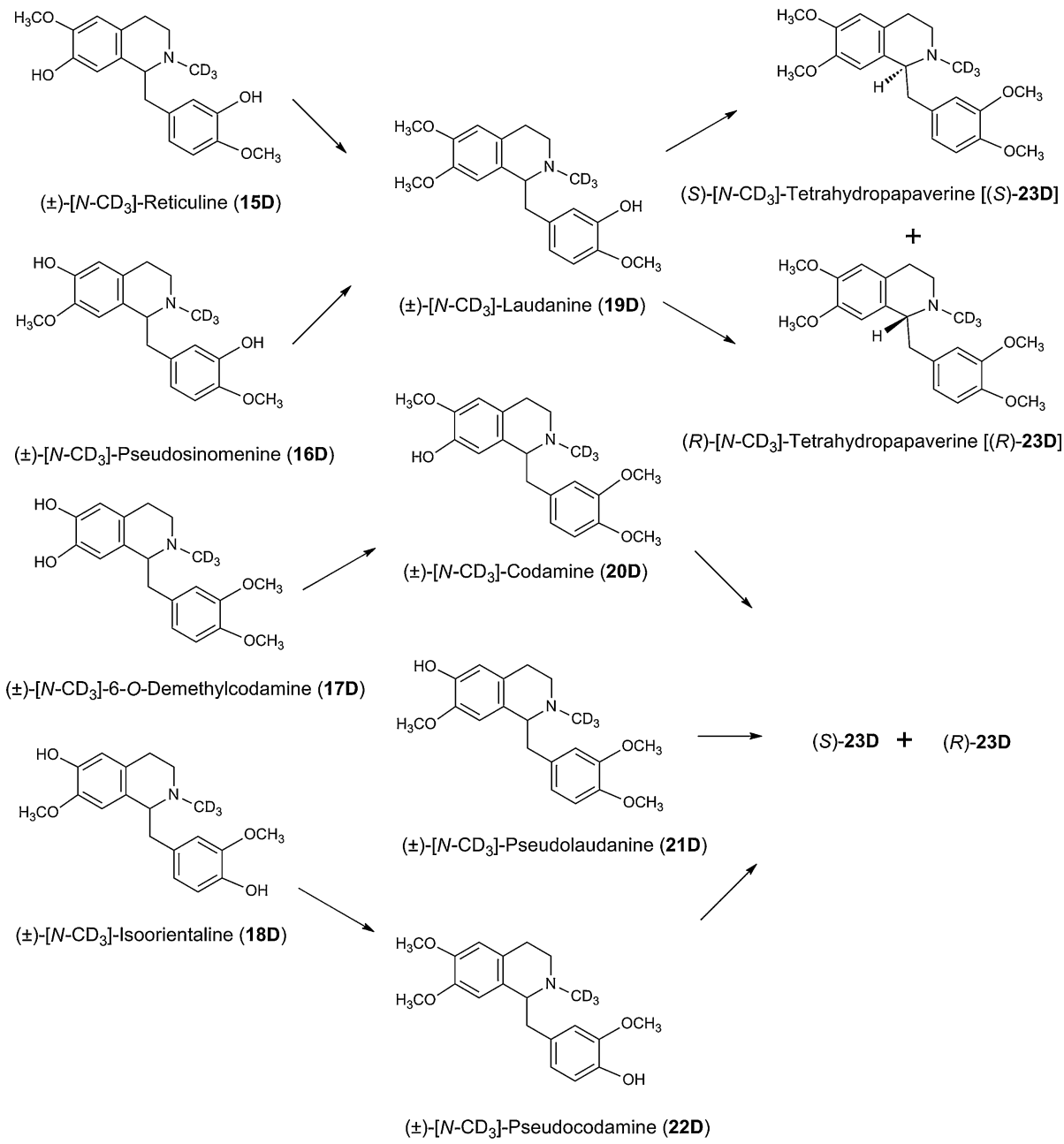
So far the results obtained from ground parts of the whole plant. – The extracts of calli of *N. domestica* showed the presence of (*R*)-carnegine **(R-11)**. Comparison with the LC-CDs of (*R*)- and (*S*)-carnegine proved the carnegine in these calli to be of (*R*)-configuration. Berberine **(12),** jatrorrhizine **(13),** and palmatine **(14)** were also found. This is reasonable for a Berberidaceae species (Scheme 2).



Scheme 2: Terrestribisamides (**10**) and their proposed biosynthetic pathway in *Nandina domestica* and isoquinoline alkaloids (**11–14**) in calli of *Nandina domestica*

Whilst the occurrence of this simple tetrahydroisoquinoline **11** fits to the incidence of more complex isoquinolines mentioned above, the terrestribisamides show a branch-line already at the point of tyrosine (see Scheme 4, below). Also in the calli cultures, E,E-, E,Z- and Z,Z-terrestribisamides **10** were detected, identified *inter alia* by a 12 Hz coupling constant of the Z diastereomers **E,Z-10** and **Z,Z-10**. It is remarkable that the other alkaloids, found in the whole plant, did not occur in the calli, and that no protopines and – consequently – no benzophenanthridines (see below, Scheme 5) were biosynthesized, the quaternary protoberberines **12** – **14** (Scheme 2), however, were identified.

Iwasa et al. (2009) offer a short overview of enzymes involved in the biosynthesis of 1-benzylisoquinoline derived alkaloids (Gerardy and Zenk 1993) and of on-line LC-CD (Bringmann et al. 1999). The references cited by Iwasa et al. (2009) enable research groups to get familiar with all these aspects. Already the title of this paper is a short summary of studies concerning the stereospecificity of the formation of metabolites in matrix extracts and calli of *Corydalis platycarpa, Macleaya cordata,* and *Nandina domestica.* Because methylations are investigated, in all the experiments N-CD_3- labeled molecules (Scheme 3; see below) were administered in order to get clear-cut results. In a structured chart, Iwasa et al. (2009) show the enrichment of alkaloidal fractions and separation from non-basic substances. As a reference for CD-measurements, racemic reticuline (**15D**; D symbolizes N-CD_3) and its (+)-rotating enantiomer were used, which has S-configuration at C-1 (Brochmann-Hansen and Nielsen 1965). This enantiomer, showing a positive CD signal, elutes faster than the R-enantiomer on a specified chiral column. This result was transmitted to the strictly related 1-benzyltetrahydroisoquinolines under investigation of O-methylation. To find out the stereospecificity of the O-methylases involved, the R/S-ratios were determined by making use of the intensity of



Corydalis platycarpa: Biotransformations of (±)-**15D** - (±)-**22D** into (*R*)- and/or (*S*)-**23D**;
Macleaya cordata: Biotransformations of (±)-**19D**, (±)-**20D**, and (±)-**22D** into (*R*)- and/or (*S*)-**23D**
Nandina domestica: Biotransformations of (±)-**15D**, (±)-**16D**, (±)-**19D**, and (±)-**22D** into (*R*)- and/or (*S*)-**23D**

Scheme 3: *O*-Methylations of phenolic [N-CD_3]-l-benzylisoquinokines {(±)-**15D** -(±)-**22D**} in plant tissue cultures

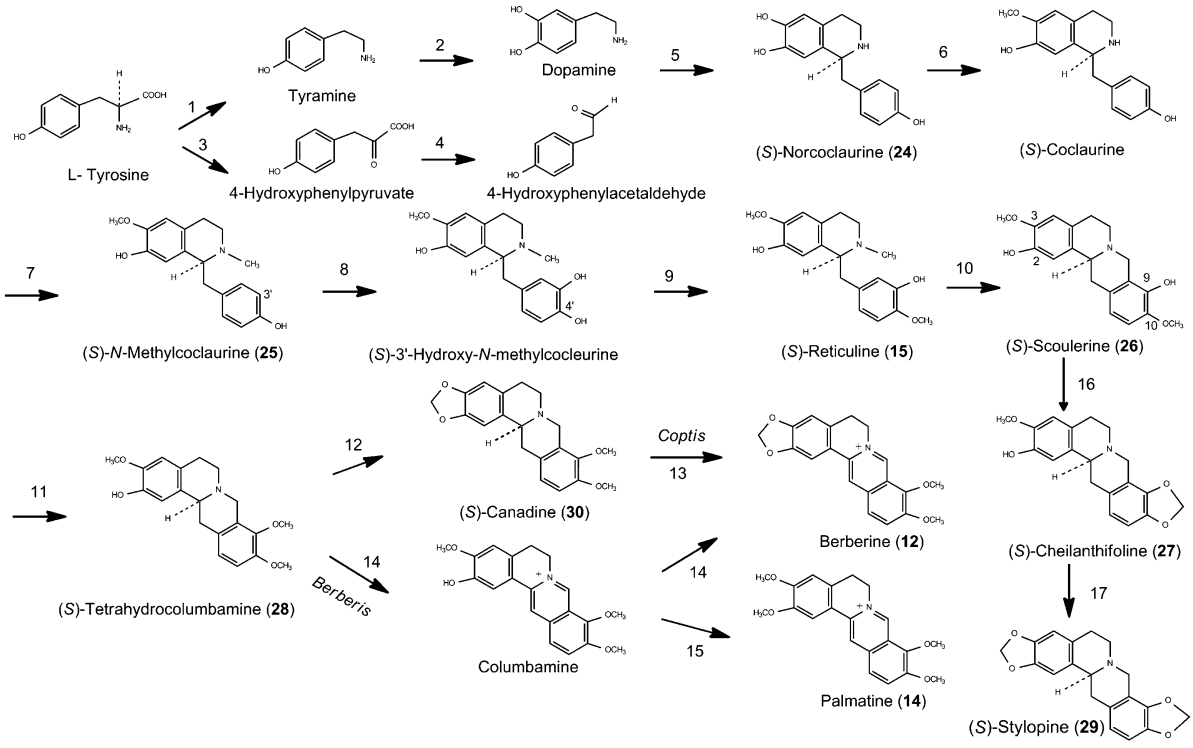
peaks in UV-, LC-, CD- and LC-MS-spectra. The optical purities of the peaks range from 89–99%, indicating the precision of separations achieved under the conditions cited.

In detail, these conditions were applied to experiments with *Corydalis platycarpa* (Fumariaceae), *Macleaya cordata* (Papaveraceae), and *Nandina domestica* (Berberidaceae), contributing the racemates of the phenolic 1-benzyl-N-methyl-tetrahydroisoquinolines rac. **15D – 22D** to cell cultures of *C. platycarapa, M. cordata,* and *N. domestica* (see Scheme 3 and legend). As already indicated, both enantiomers were utilized in these plants to produce O-methylated metabolites. High enantioselectivity of the O-methyltransferase was found in **22D** by administration of (*R*)- and (*S*)-enantiomers of **19D**, **21D**, and **22D** to cell cultures of *C. platycarpa.*

3. Biosynthesis of protoberberines and related alkaloids

In nature most protoberberines are found as tetrahydro derivatives. Dehydrogenation generates quaternary protoberberinium salts. Dihydroprotoberberines are rare due to their sensitivity, resulting from their enamine increment. *N*-Oxides of the tetrahydroprotoberberines (Tani et al. 1975b) and quaternary N-methyl-tetrahydroprotoberberines are known. In general O-substituents are located at C-2, C-3, C-9, and C-10 (protoberberines), whilst oxygenation at C-2, C-3, C-10, and C-11 generates *pseudo*protoberberines (nomenclature according to Preininger 1986) (Scheme 6, see below).

Oxygenation at C-14 in addition to N-methylation are prerequisites for the formation of protopine alkaloids which are precursors of benzophenanthridine-, rhoeadine-,



1: L-tyrosine decarboxylase
2: Phenolase
3: L-Tyrosine transaminase
4: *p*-Hydroxyphenylpyruvate decarboxylase
5: (*S*)-Norcoclaurine synthase (**I**)
6: Norcoclaurine- 6-*O*-methyltransferase (6-OMT) (**II**;**III**)
7: Coclaurine *N*-methyltransferase (NMT) (**IV**)
8: (*S*)-*N*-Methylcoclaurine 3'-hydroxylase (**V**)
9: (*S*)-3'-Hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (**VI**)
10: Berberine bridge enzyme (BBE) (**VII**)
11: (*S*)-Scoulerine 9-*O*-methyltransferase (SMT) (**VIII**)
12: (*S*)-Canadine synthase (**IX**;**X**)
13: (*S*)-Canadine oxidase (COX) (**XI**)
14: (*S*)-Tetrahydroberberine oxidase (includes methylenedioxyring forming enzyme) (**XII**;**XIII**)
15: Columbamine *O*-methyltransferase (**XIV**)
16: (*S*)-Cheilanthifoline synthase (**XV**)
17: (*S*)-Stylopine synthase (**XVI**)

I: Minami et al. (2007); **II**: Sato et al. (1994); **III**: Inui et al. (2006); **IV**: Choi et al. (2002); **V**: Pauli and Kutchan (1998); **VI**: Morishige et al. (2000); **VII**: Kutchan and Dittrich (1995); **VIII**: Dubouzet et al. (2005); **IX**: Ikezawa et al. (2003); **X**: Rueffer and Zenk (1994); **XI**: Yamada and Okada (1985); **XII**: Amann et al. (1988); **XIII**: Galneder et al. (1988); **XIV**: Morishige et al. (2002); **XV**: Bauer and Zenk (1991); **XVI**: Ikezawa et al. 2007.

Scheme 4: Biosynthesis of protoberberines from L-tyrosine, cf. Kuthcan (1998); Croteau et al. (2000); Bauer and Zenk (1991); Sato et al. (2007)

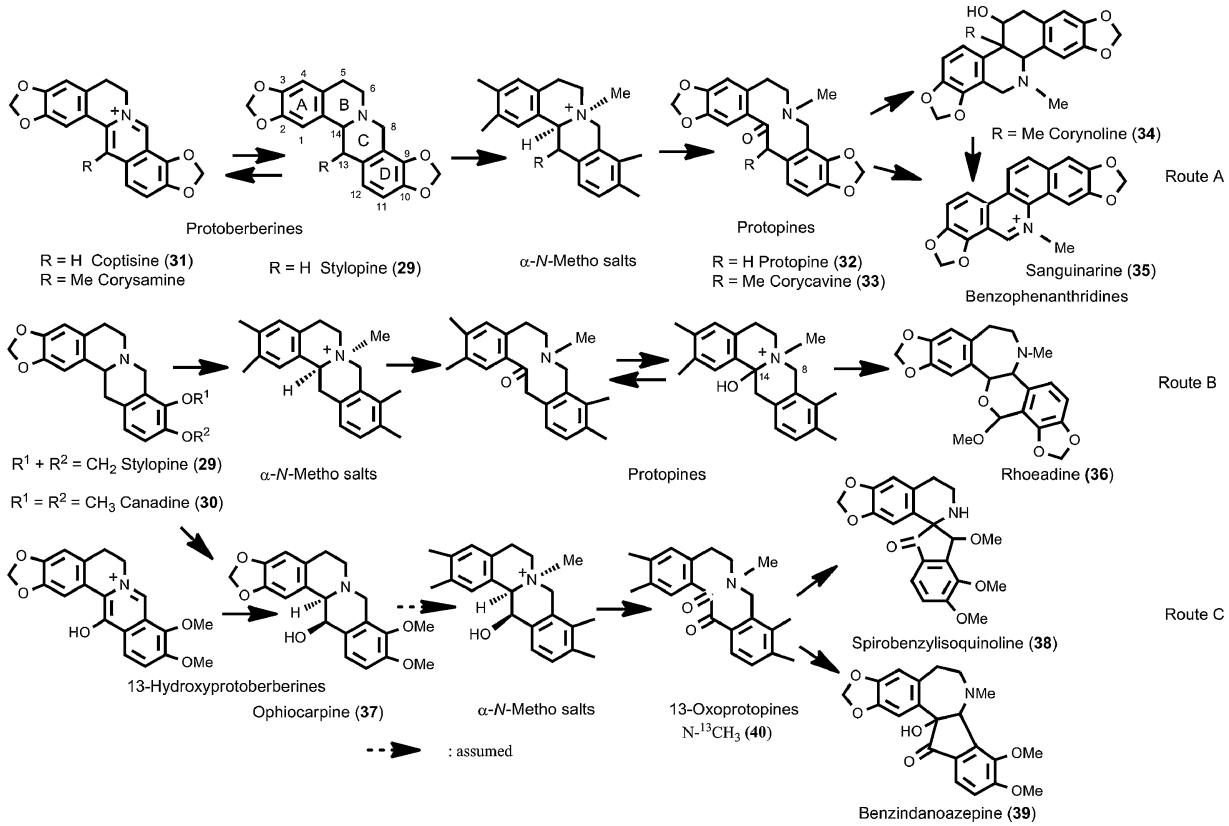
spirobenzylisoquinoline-, and benzindanoazepine-alkaloids (Scheme 5, see below).

Valuable insight into the biosynthesis of protoberberines with L-tyrosine (Gear and Spenser 1963) and dopamine (Monkovic and Spenser 1964) as educts (Scheme 4) also stems from feeding experiments, i.e. by administration of potential precursors to whole plants and to corresponding cell cultures (Bauer and Zenk 1991; Kutchan 1998; Croteau et al. 2000).

According to Beecher and Kelleher (1988) L-tyrosine molecules are converted into two C_6-C_2-components which condense to (*S*)-norcoclaurine **(24)** which is converted to (*S*)-*N*-methylcoclaurine **(25)** (Scheme 4). According to Mueller and Zenk (1992) protoberberines can be biosynthesized by introduction of the fourth O-function at a late stage, i.e. into the preformed 1-benzyl-*N*-methyl-tetrahydroisoquinoline. Thus, (*S*)-*N*-methylcoclaurine **(25)** is hydroxylated at C-3'(Pauli and Kutchan 1998) generating a precursor of (*S*)-reticuline **(15)**. The connection of N and the ortho-position of the benzyl increment by CH_2 (so called "berberine bridge") stems from N-CH_3 of reticuline **(15)** which is oxidized to >N-CH_2-OH, and this halfaminal splits off water thus generating the electrophilic $(>N=CH_2)^+$ group (Barton et al. 1963; Battersby et al. 1963). (*S*)-Reticuline **(15)** is cyclised to (*S*)-scoulerine (**26**) (Kutchan and Dittrich 1995). The phenolic groups of scoulerine are O-methylated or transformed to methylenedioxy rings. Thus, (*S*)-cheilantifoline (**27**) which is methylenedioxy-substituted at ring D of its skeleton, is generated (Bauer and Zenk 1991), whilst the phenolic OH of (*S*)-scoulerine **(26)** at C-9 is methylated to (*S*)-tetrahydrocolumbamine (**28**) (Amann et al. 1988; Galneder et al. 1988), which was converted to palmatine **(14)** *via* columbamine (Kutchan and Dittrich 1995; Bauer and Zenk 1991). (*S*)-Stylopine (**29**) and (*S*)-canadine (**30**) are formed analogously (Amann et al. 1988; Galneder et al. 1988). Berberine (**12**) was prepared from (*S*)-tetrahydrocolumbamine (**28**) in *Berberis* species and (*S*)-canadine (**30**) by *Coptis* species (Scheme 4) (Amann et al. 1988; Galneder et al. 1988; Yamada and Okada 1985).

The evidence for three different pathways proceeding from protoberberines (Scheme 5) has been reviewed (Iwasa 1995a). The conversion of (non-phenolic) protoberberines into protopines (Takao et al. 1976; Iwasa et al. 1993a), benzophenanthridines (Kutchan and Dittrich 1995; Battersby et al. 1975; Iwasa et al. 1993a, 1988, 1989; Battersby et al. 1979; Takao et al. 1983; Zenk 1994), rhoeadines (Roensch 1972, 1977, 1986; Battersby and Staunton 1974; Tani and Tagahara 1977), benzindanoazepines (Iwasa et al. 1984a, 1984b, 1985), and spirobenzylisoquinolines (Iwasa et al. 1984a,b, 1985) (Scheme 5) has been studied.

These biogenetic routes (Scheme 5, routes A – C) proceeding from protoberberines were found by feeding experiments with whole plants and by addition to callus cultures of various species. The following list shows the families and species concerned together with some corresponding literature references.



Scheme 5: Biosynthetic routes from protoberberines to other skeletal alkaloids

Fumariaceae

Corydalis incisa: Tani and Tagahara (1974); Takao et al. (1976); Yagi et al. (1977); Iwasa et al. (1988, 1989, 1993a, 1995a).
C. ochotensis var. *raddeana*: Iwasa et al. (1985, 1988)*;* Iwasa and Kamigauchi 1996.
C. ophiocarpa: Iwasa and Takao (1982); Jeffs and Sharver (1976); Iwasa et al. (1985 1988); Iwasa and Kamigauchi (1996).
C. pallida var. *tenuis*: Iwasa et al. (1988, 1994, 1995b)
C. platycarpa: Iwasa and Takao (1982); Iwasa et al. (1985, 1988).
Dicentra spectabilis: Iwasa and Kim (1997).

Papaveraceae

Chelidonium majus: Leete and Murril (1967); Takao et al. (1976); Battersby et al. (1975, 1979)
Macleaya cordata: Takao et al. (1983).
Papaver bracteatum: Roensch (1972, 1977).
Papaver rhoeas: Battersby and Staunton (1974); Tani and Tagahara (1977).

Scheme 5 describes the sequence: protoberberinium salts (e.g. coptisine **31**), reduction to their tetrahydro derivatives (e.g. stylopine **29**; analogous for 13-methyl-protoberberinium salts), N-methylation affording α-N metho salts (rings B and C *cis* configurated) (Takao et al. 1976, 1983; Iwasa et al. 1993a) and C-14 hydroxylation (Takao et al. 1976; Iwasa and Takao 1982; Iwasa et al. 1993a, 1985) to protopines (e.g. protopine **32** and corycavine **33**). These quaternized halfaminals open the C-14 - N-bond, resulting in a 10-membered achiral compound, followed by C-6 hydroxylation (Iwasa et al. 1989) and rearrangement to benzophenanthridines (corynoline **34**, sanguinarine **35**, e.g.). Detailed mechanistic explanations are shown in Iwasa (1995a).

The pathway of Route A in Scheme 5 was also demonstrated based on enzyme-level studies in several cell cultures by Zenk's (1994) and Sato's (2007) groups.

Papaveraceae

Eschscholzia californica: Tanahashi and Zenk (1988, 1990); Schumacher and Zenk (1988); De-Eknamkul et al. (1992); Kammerer et al. (1994); Inui et al. (2007); Ikezawa et al. (2007, 2009); Takemura et al. (2010).

Berberidaceae

Berberis wilsoniae: Amann et al. (1988); Zenk (1985).

Fumariaceae

Corydalis vaginans: Rueffer and Zenk (1987).
Dicentra spectabilis: Iwasa and Kim (1997).
Fumaria capreolata: Rueffer and Zenk (1986).

Ranunculaceae

Coptis japonica: Mueller and Zenk (1992); Galneder et al. (1988); Yamada and Okada (1985); Ikezawa et al. (2003); Sato et al. (1994); Choi et al. (2002); Morishige et al. (2000, 2002); Dubouzet et al. (2005); Minami et al. (2007).
Thalictrum bulgaricum: Kammerer et al. (1994).
Thalictrum tuberosum: Rueffer and Zenk (1994).

Route B shows the conversion of protopines to rhoeadines (Scheme 5, middle). By comparison of the corresponding structures it becomes evident, that the C-8-N and the C-14-N bond

have to be split in order to widen the tetrahydroisoquinoline part of the protoberberines into the benzo[*d*]azepine system (Iwasa 1995a).

Moreover, six oxygen functions in rhoeadines as compared to four in tetrahydroberberines point towards hydroxylations. A tetrahydroprotoberberine is N-quaternized and hydroxylated at C-14 and C-8, thus forming two half-aminals which provoke two C-N- cleavages. Then recyclizations generated the azepine ring of rhoeadine alkaloids (Iwasa 1995a).

Route C (Scheme 5, bottom) compiles the conversion of 13-hydroxyprotoberberines to spirobenzylisoquinolines (**38,** e.g.) and benzindanoazepines (**39**, e.g), resp. Hydroxylation at C-13 of tetrahydroberberines (**30**, e.g.) generates *cis*-13-hydroxy derivatives (**37**, e.g.). The corresponding diastereomer may result from **30** by removal of the *pro*-13S hydrogen. This follows from reactions of C-13 tritiated tetrahydroberberines (Jeffs and Sharver 1976). Both diastereomers are N-methylated generating the corresponding α-N-metho salts. Hydroxylation at C-14 opens the quaternary half-aminal to the ten-membered ring which is oxidized to the corresponding 1,2-diketone (a 13-oxo-protopine). Some rearrangements lead to spirobenzylisoquinolines (**38**, e.g.) and benzindanoazepines (**39**, e.g.; Iwasa et al. 1985). Feeding experiments with **40** (labelled N-Me* groups) indicate that this Me* generates the O-Me* increment at the benzylic position of **38**.

According to Preininger (1986) 2,3,10,11 oxygenated protoberberines are called *pseudo*protoberberines. In biological tests some of these alkaloids show higher efficacy than the 9,10-oxygenated regioisomers. – There arose the question whether these 2,3,10,11-oxygenated protoberberines are metabolized analogously to the more frequently occurring 2,3,9,10-regioisomers. To tackle this issue, LC-NMR and LC-MS measurements were performed (Iwasa et al. 2003).

Whilst the biosynthesis of protoberberines has been thoroughly studied (Iwasa 1995a; review), there were no corresponding experiments dealing with pseudoprotoberberines. Therefore, 2,3,10,11-methylenedioxy- and methoxy-substituted pseudotetrahydroprotoberberines, some of them with a dehydrogenated ring C, were administered to cell cultures of *Corydalis ochotensis var. raddeana* and *C. platycarpa* (Iwasa et al. 2003) which proved to metabolize protoberberines effectively. By LC-NMR alkaloidal metabolites were found without isolation, thus targeting their isolation by HPLC. Here, preceding experiments of Wolfender et al. (1998, 2001) helped a lot in analyzing extracts of cell cultures. – Two tetrahydropseudoprotoberberines were prepared according to Lenz (1977). N-Methylation of each one led to a mixture of α- and ß-metho salts (*cis*- or *trans*-quinolizidine systems, resp.). Deuterated metho salts were obtained by CD_3I. A detailed chart (Iwasa et al. 2003) shows work-up of the calli and the (agar) medium. NMR- and MS data of the compounds under consideration are listed. LC-APCI-MS with SIM and TIM were measured in the positive ion mode, LC-NMR spectra in the stop-flow mode (Smallcombe et al. 1995). When 2,3,10,11-bismethylenedioxy-tetrahydropseudoprotoberberine (tetrahydropseudocoptisine; **41;** Scheme 6) was administered, the corresponding NMR-spectrum of one fraction showed the α-N-metho salt of tetrahydropseudocoptisine **(42)** (*cis* configuration of rings B and C). By comparison with authentic samples of α- and ß-N-metho salts, its α configuration was established. Moreover, the dehydrogenated (ring C) pseudoprotoberberine (pseudocoptisine **43**) occurred, and some remaining starting material was detected.

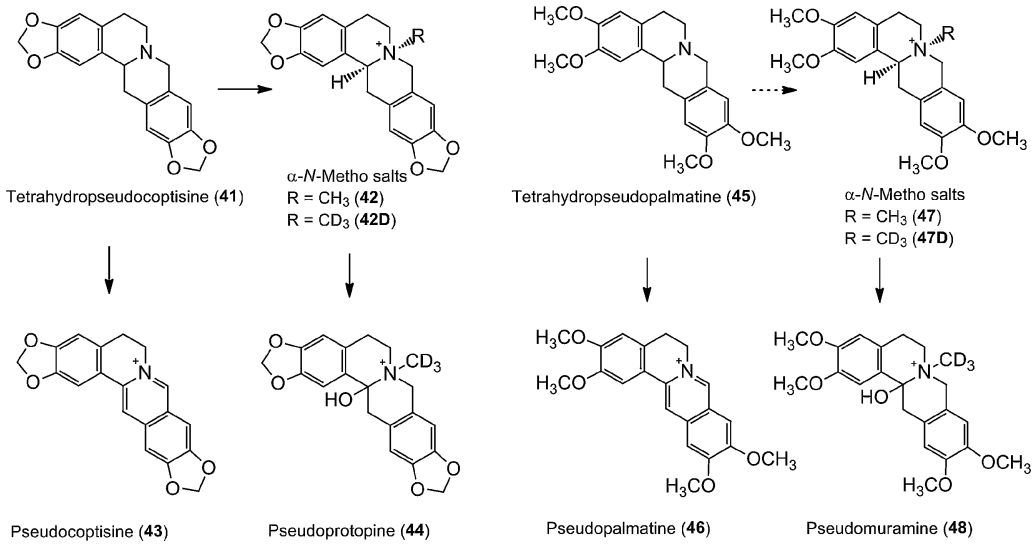
When calli of two different *Corydalis* species were fed with the N-CD_3- tetrahydropseudocoptisine α-N-metho salt **42D** for up to 28 days, the corresponding pseudoprotopine **44** arose, indicating oxygenation at C-14 of the starting material. – Remarkably, results obtained using tetrahydropseudopalmatine (**45)** were only partially analogous to those achieved when using the corresponding methylenedioxy-alkaloid. Pseudopalmatine (**46**) arose by dehydrogenation, some of its tetrahydro derivative **45** remained unchanged, but no N-methyl derivative **47** and consequently no pseudoprotopine-type alkaloid was found. When [N-CD_3]-tetrahydropseudopalmatine-α-N-metho salt (**47D**), however, was administered, the corresponding pseudoprotopine type alkaloid pseudomuramine (**48**) came up by oxygenation of C-14. This can be explained by assuming a high substrate specificity of the N-methyl transferase. In conclusion: tetrahydropseudocoptisine-α-N-metho salt **42**, the appropriate pseudoprotopine **44** – both with two methylenedioxy increments – and pseudomuramine (**48**), related to pseudopalmatine (**46**), are new alkaloids, thus pointing towards the fact that *Corydalis* species possess enzymes suitable for the synthesis of pseudoprotoberberines in accordance with Preininger (1986).

It is considered that 2,3,9,10- and 2,3,10,11-oxygenated protoberberines branch at the stage of 1-benzylisoquinoline alkaloids. Therefore, the conversion of 1-benzylisoquinolines into 2,3,10,11-oxygenated protoberberines was studied. In this context, we examined a possible influence of a phenolic OH group at the rings A and/or D of 1-benzyl-tetrahydroisoquinolines. Thus, callus cultures of *Macleaya cordata, Corydalis platycarpa* and *C. chotensis var. raddeana* were incubated with mono-phenolic 1-benzyl-tetrahydro-isoquinolines, obtained by acid-catalyzed cleavage of methoxy groups of tetrahydropapaverine (**49**) (Iwasa et al. 2005).

When 3'-hydroxy-4',6,7-trimethoxy-1-benzyltetrahydroisoquinoline (**50**) was administered to *C. ochotensis var. raddeana*, **51** and **52** were found. In **50** the formation of the methylene bridge ortho/para to the phenolic OH occurs, producing 9-hydroxy-2,3,10-trimethoxy-tetrahydroprotoberberine (**51)** and the regioisomeric pseudotetrahydroprotoberberine **52** (term according to Preiniger 1986), in which the OH group is located at C-11. Here the phenol attacks the preceeding $(>N=CH_2)^+$ increment *via* the para position. The protopine-type alkaloid **55** was produced from **50**, indicating the formation of a methylenedioxy group arising from the phenolic OH and the neighboring methoxy group, the formation of a methylene bridge between the ortho position (C-2') of the phenolic increment and the N-atom, N-methylation and oxygenation at C-14 (Scheme 7).

Dehydrogenation of **51** (combined with the formation of a methylenedioxy group) and **52** produced the protoberberinium and pseudoprotoberberinium salts **56** and **54**, resp. – When the phenolic alkaloid **50** was fed to *Macleaya cordata*, the metabolites **51**, **52**, **54**, and **53** were also produced. In *Macleaya cordata,* remethylation of OH to OCH_3 in **50** occurred together with dehydrogenation of ring B, as indicated by the formation of papaverine (**53**). – The phenolic alkaloid **50**, when fed to *C. platycarpa* was also bioconverted to the protopine-type alkaloid **55**, to the tetrahydropseudoprotoberberine **52** and to the tetrahydroprotoberberine **51**, which are identic with the metabolites generated in *C. ochotensis* var. *raddeana* and in *M. cordata.* The phenolic alkaloid **50** was converted to the N-methylated derivative **19** which is the prerequisite for the formation of the dibenzo[*d,g*]quinolizidines **51** and **52** (see above).

Striking differences arose when the regioisomers of **50**, 1-benzyl-4'-hydroxy-3',6,7-trimethoxy-1,2,3,4-tetrahydroisoquinoline (**59**) or 1-benzyl-1,2,3,4-tetrahydroisoquinolines with an OH group at ring A, **57** and **58**, resp., were metabolized by *C. platycarpa* calli: no tetrahydroprotoberberines or tetrahydropseudoprotoberberines arose. These potential precursors were only N-methylated to **20**, **21**, and **22**. As has been pointed out, the phenolic 1-benzyltetrahydroisoquinolines had been obtained from rac. tetrahydropapaverine **(49;** Scheme 7). Therefore, the



Scheme 6: Bioconversions of pseudoprotoberberines into pseudoprotopines; broken line: assumed

stereochemistry of the metabolites had to be clarified. According to Kametani's (1968) classical X-ray structure analyses of levo-rotating tetrahydroprotoberberines it is evident, that these levo-rotating alkaloids have (*S*)-configuration at C-14. The enantiomers of tetrahydropalmatrubine (**51**), e.g., were separated on chiral columns by preparative HPLC. Because the positions of the substituents at the rings A and D do not or only scarcely influence the specific rotation at the sodium D line, we used the LC-CD spectra of tetrahydroprotoberberines also for the determination of configurations of pseudotetrahydroprotoberberines (Jeffs 1967).

The configurations at C-14 were determined by optical rotation, and with these data at hand, the enantiomeric composition of **52** and **51** were determined by LC-CD. While the phenolic 1-benzyl-tetrahydroisoquinoline **19** (Scheme 8) was mainly converted to (*S*)-**52** by calli of *M. cordata*, (*R*)-**51** is dominating in the mixture of **51** enantiomers. This situation is parallel to that found with calli of *C. platycarpa* and *C. ochotensis* var. *raddeana*. – Besides analytical progress described there, this was the first report on the bioconversion of a 1-benzyl-tetrahydroisoquinoline into a 2,3,10,11-oxygenated pseudoprotoberberine in callus cultures.

The results documented in the following paragraph are related to those discussed: we compared the metabolism of NH-1-benzyl-tetrahydroisoquinolines (see above) with analogous N-CH_3 derivatives (Cui et al. 2007). Moreover, some 1-benzyl-tetrahydroisoquinolines have one, others two phenolic OH groups. N-CD_3 analogues were also administered to cell cultures of *C. platycarpa, C. ochotensis* var. *raddeana,* and *M. cordata* in order to differentiate between metabolites of the incubated molecules and those which might come up by independent processes. The metabolic conversions are summarized in Scheme 8.

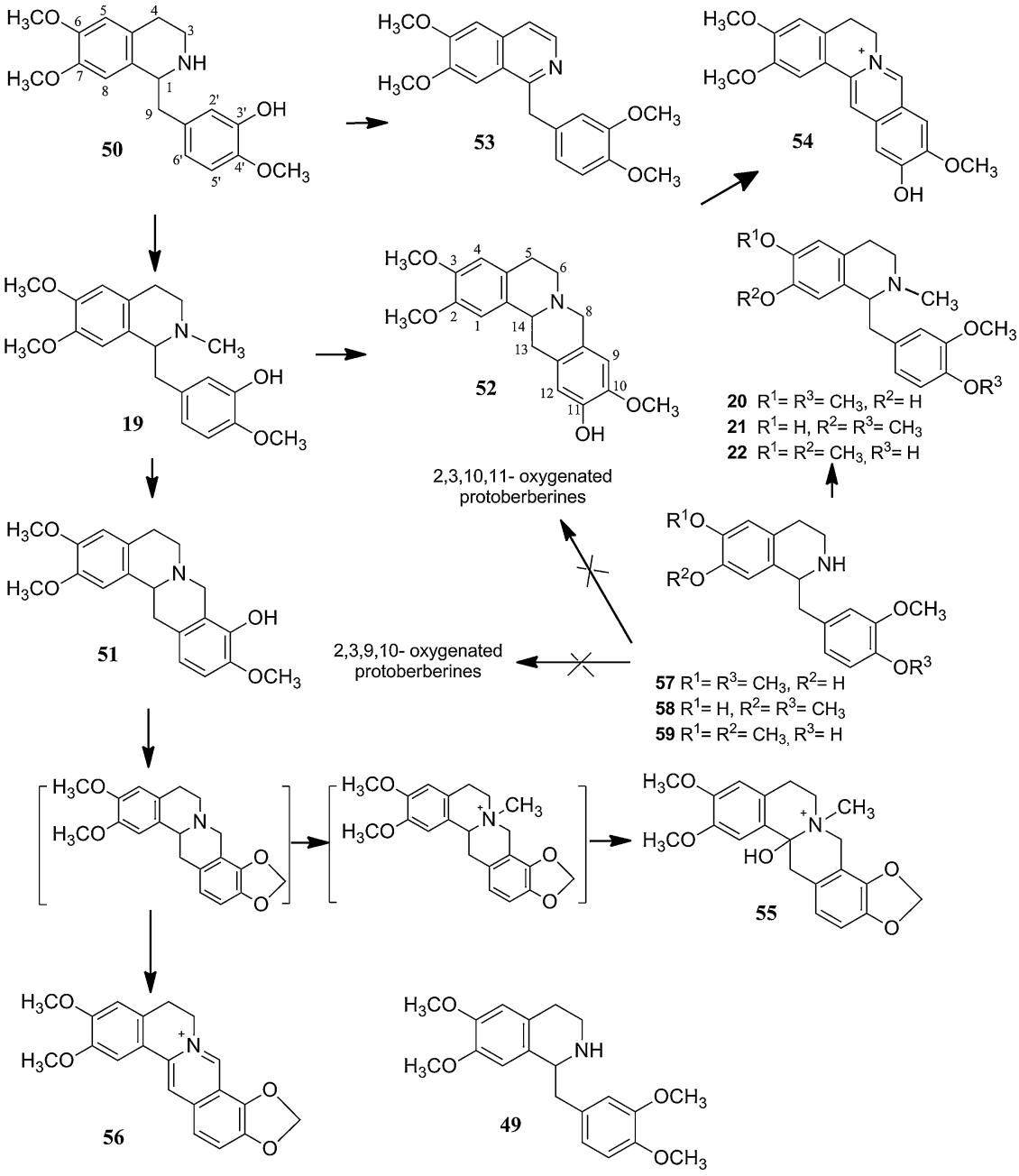
The mono-phenolic 1-benzyl-tetrahydroisoquinoline **19,** carrying OH at C-3', forms a bridge between C-2'or C-6', using the N-CH_3 increment (berberine bridges in **51** and **52**). This is analogous to the bioconversion of 1-benzyl-3'-hydroxy-4',6,7-trimethoxy-1,2,3,4-tetrahydroisoquinoline (**50;** Scheme 7), also producing a berberine bridge achieving the 2,3,9,10-oxygenated tetrahydroprotoberberine **51** and – in addition – the 2,3,10,11-oxygenated regioisomeric tetrahydropseudoprotoberberine **52** (Iwasa et al. 2005). Obviously there is a preceding N-methylation. Tetrahydropalmatrubine (**51**) was converted into tetrahydroepiberberine (**60**), which was further metabolized into cryptopine (**55**) and epiberberine (**56**) (Scheme 8). Obviously, N-methylation overcomes the deviating metabolism, which apparently seems to be due to the difference N-H *versus* N-CH_3. – Bis-phenols (Cui et al. 2007) were administered to the callus of *C. platycarpa.* O-Methylation in the bisphenol reticuline **15** (OH groups at C-3′ and C-7) to the mono-phenol **19** occurred as a minor pathway. Rather, **15** was converted to the tetrahydroprotoberberine (*S*)-**26**, whilst in the bisphenol **16** (OH groups at C-3′ and C-6) C-6-OH was methylated to O-CH_3 of **19**. Monophenol **19** was transformed into **52** and **51** which was converted to cryptopine (**55**) v*ia* tetrahydroepiberberine (**60**).

All precursors had been administered as racemates in order to study stereochemical aspects of bioconversion. As a results: the tetrahydroberberines mentioned above are S-configurated at C-14 as shown by LC-CD in comparison with (*S*)-scoulerine (**26**). This points to stereospecificity of the converting enzyme(*s*).

Also the youngest project of our group (Iwasa et al. 2010) deals with the comparison of biochemical conversions of protoberberines and their *pseudo*-regioisomers in *Macleaya cordata (M.c.), Corydalis ochotensis* var. *raddeana (C.o.),* and *Nandina domestica (N.d.).* In preceding paragraphs we have shown that an OH-group at C-3′ of 1-benzyl-tetrahydroisoquinolines is a prerequisite for the formation of the berberine bridge (Iwasa et al. 2005; Cui et al. 2007). For NH-tetrahydroisoquinolines, N-methylation is a preceding reaction, and the ring closure can come up ortho to the phenolic OH, leading to tetrahydroprotoberberines, or para to this electron donating increment, as indicated above, thus generating the corresponding tetrahydropseudoprotoberberines.

In this context, the bioconversion of the tetrahydropseudoprotoberberines **52**, **61**, and **62** which carry OH-groups at their rings D in 10 and/or 11-position, were studied in comparison with 9-hydroxy-2,3,10-trimethoxy-tetrahydroprotoberberine (**51**).

In order to avoid misinterpretation of enzymatic methylation of phenolic OH groups to OCH_3 increments, of oxygenation at C-8, of dehydrogenation of ring C, of N-methylation, followed by conversion to pseudoprotopines, and of formation of a methylendioxy increment from a phenolic OH and the neighboring methoxy group, the substrates used for incubation were labeled with ^{13}C or deuterium at C-8. Consequently, suitable 1-benzyltetrahydroisoquinolines were reacted with ^{13}C-formaldehyde or CD_2O in a Mannich reaction. The conversions by the plants mentioned above are compiled in Scheme 9.



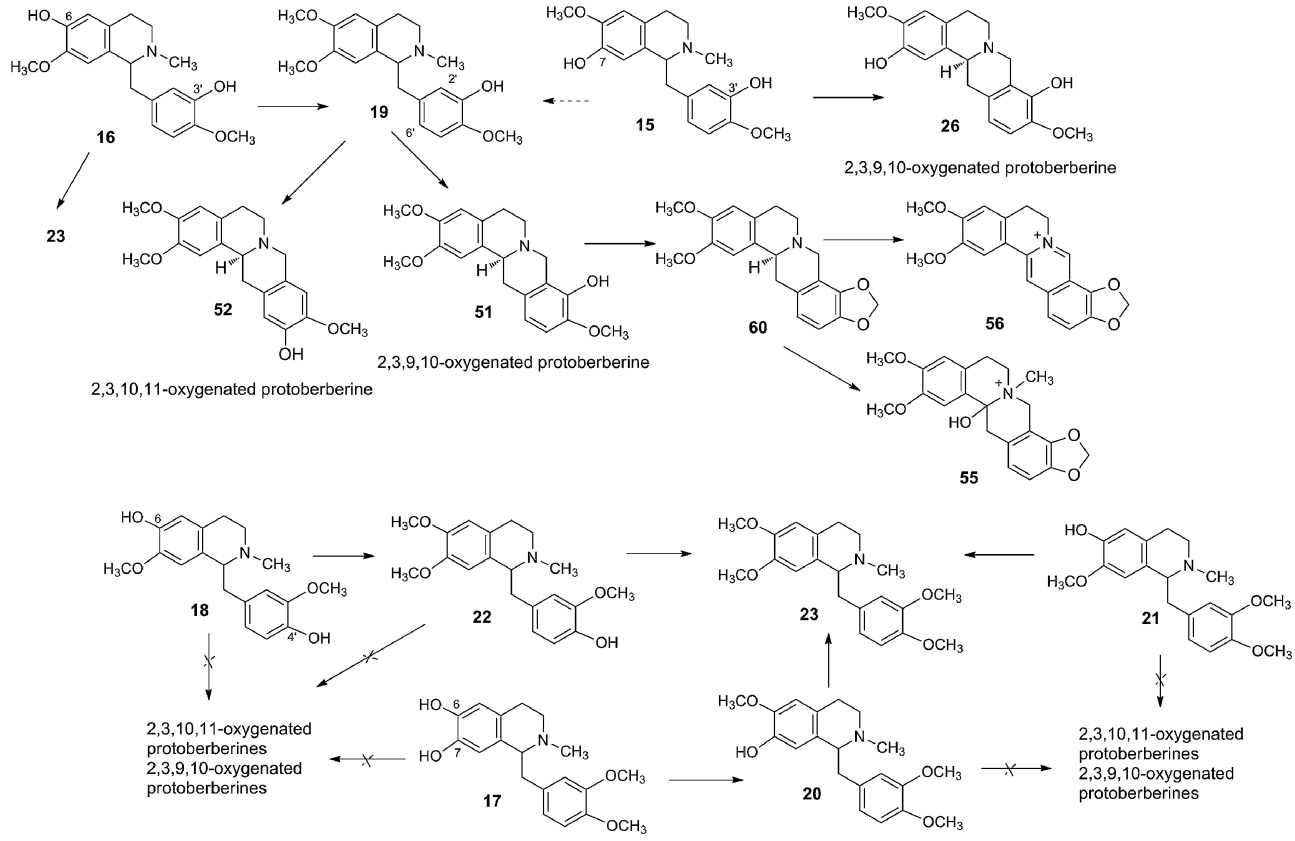
Scheme 7: Metabolism of phenolic 1-benzylterahydroisoquinolines

For the elucidation of the metabolic reactions, the racemates of **51**, **52**, **61**, and **62**, ^{13}C-labelled at C-8, and rac. **62**, deuterated at C-8, were used for experiments with *N. domestica* (N.d.), *C. ochotensis* var. *raddeana,* (C.o.), and *M. cordata* (M.c.). Rac. **62** was O-methylated at C-10-OH and C-11-OH stereospecifically, yielding (*S*)-**52** and (*S*)-**61,** resp.

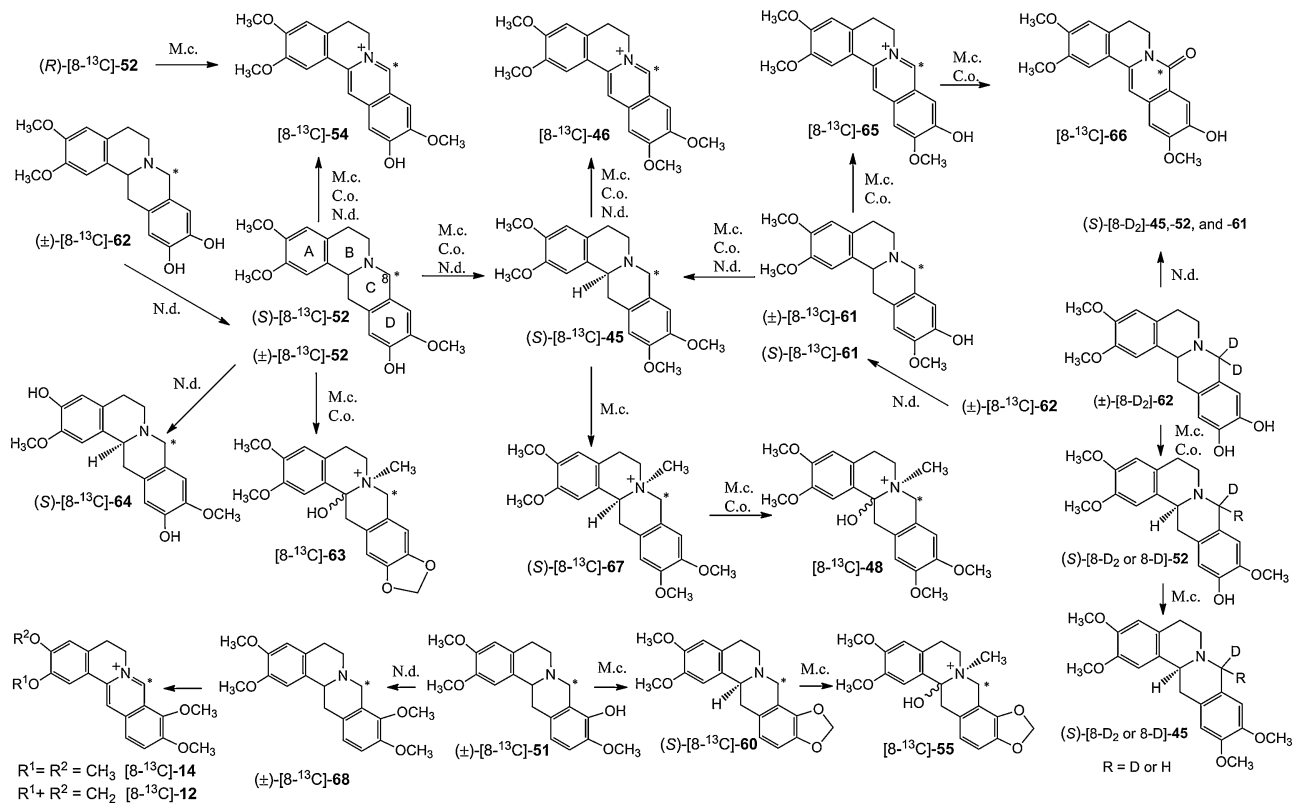
Only N.d. cleaved the C-3-methoxy group of rac. **52**, leading to the diphenol coreximine (*S*)-**64**. Obviously, O-methylation and O-demethylation in N.d. are stereospecific. (*S*)-**52** was converted to the methylenedioxy-substituted alkaloid pseudocryptopine **63** by M.c. and C.o. This stereospecificity was also observed in the conversion of rac. **52** into (*S*)-**45** by all three plants. Identically, (*S*)-**45** was generated from rac. **61** by these plants. This holds true also for the calli of these three plants which convert rac. **61**, a regioisomer of **52,** of which OCH_3 and OH at C-10 and C-11 of ring D are exchanged, to (*S*)-**45**. Here, methylenedioxy-formation at ring D was not observed. – Dehydrogenations of **52, 45,** and **61** led to the pseudoprotoberberines **54**, **46**, and **65**, resp. – Compounds **54** and **46** were obtained from the calli of the three plants under consideration, whilst **65** was generated by M.c. and C.o. only. Compound **65** was oxygenated by these two plants to the lactam **66**.

(*S*)-**45** was N-methylated to the quaternary salt **67** with *cis*-configurated rings B and C, a preliminary stage of (achiral) pseudomuramine (**48**). Last but not least, rac. **62** was transformed by N.d. to the (*S*)-enantiomers of **52**, **61,** and **45**, and M.c. and C.o. methylated C-10-OH of rac. C-8 deuterated **62** stereospecifically to (*S*)-**52**, accompanied by partial D/H exchange. M.c. methylated (*S*)-**52** to the dimethoxy derivative **45** without affecting the S configuration.

Interestingly, (*R*)-**52** was not converted into (*R*)-**45,** but analogously to (*S*)-**52** it was dehydrogenated to **54**. – As in the regioisomer of **52**, in rac. tetrahydropalmatrubine (**51**) a methylenedioxy-increment was generated at ring D, thus forming tetrahydroepiberberine [(*S*)-**60**] which was transformed into the protopine alkaloid cryptopine (**55**). This reaction, however, did not occur when (*R*)-



Scheme 8: Metabolism of phenolic 1-benzyl-*N*-methyltetrahydroisoquinolines



Scheme 9: Metabolism of (±)-[18-^{13}C]-tetrahydropalmatrubine, -corytenchine, -10-demethylxylopinine, -spinosine and (±)-[8-D_2]-spinosine {(±)-[8-^{13}C]-**51**, **52**, **-61**, **-62**, and (±)-[8-D_2]-**61**} in *Macleaya cordata* (M.c.), *Corydalis ochotensis* var. vaddeana (C.o.), and *Nandina domestica* (N.d.)

51 was administered to the calli of *M. cordata*. – In N.d. formation of a methylenedioxy increment did not occur, and **51** was *O*-methylated to tetrahydropalmatine (**68**).

This section shows that metabolisations of 2,3,9,10-tetraoxygenated tetrahydro-protoberberines and their regioisomeric 2,3,10,11-tetraoxygenated tetrahydro-pseudoprotoberberines are more or less identical, but some deviations occur within

the cell cultures of the different plants under investigation. The metabolisms shown in Scheme 9 were shown for the first time (Iwasa et al. 2010). They are partially controversial to the data published by Roblot et al. (1978) and Queiroz et al. (1996), who found that *no* tetrahydroprotoberberines or their *pseudo*-regioisomers were generated when the precursors 1-benzyl-6,7-dimethoxy-tetrahydroisoquinolines with C-4′-OH or C-3′- and C-4′-OH were administered. We assume that the results summarized in this Chapter 3 offer some insight into the biochemical potential of Berberidaceae, Fumariaceae, and Papaveraceae plants and their calli, which are compiled in this review.

4. Simple isoquinolines

Since the early nineties we study biosynthesis and biochemical interconversions of 1,2,3,4-tetrahydroisoquinolines which according to their structure obviously are generated from dopamine and a C_1-increment (formally formaldehyde) or a C_2-increment (formally acetaldehyde). These studies (Iwasa et al. 1991) were stimulated by the fact, that 1,2,3,4-tetrahydroisoquinoline (TIQ; **69)** is markedly increased in the brain of Parkinson patients as compared with the quantity of this small alkaloid in the brain of healthy persons (Niwa et al. 1987; Kohno et al. 1986). On the other hand, 1-methyl-TIQ (**70**) is increased in the healthy brain, but decreased in the brain of Parkinson patients (Kohno et al. 1986). Thus, a connection between M. Parkinson and TIQs was suggested (Niwa et al. 1987; Kohno et al. 1986). After L-DOPA treatment, 6,7-dihydroxy-1-methyl-TIQ (**72**, salsolinol) was found in brain and urine of Parkinson patients, and there are relationships between the catechol **72** and alcoholism published in the last decades of the 20th century. – 4-Hydroxy-TIQ (**71**) was found in liver and urine of rats (Ohta et al. 1990). – Nowadays an influence on alcoholism is no longer assumed (Musshoff et al. 2005). A connection with Morbus Parkinson, however, is corroborated by Lorenc-Koci et al. (2008) and Kobayashi et al. (2009). Catechols are O-methylated by COMT. Thus salsolinol (**72**) is converted to its 7-OMe-derivative **74** (salsoline) *in vivo* in brain and heart of rats. In contrast, slices of rat liver converted **72** to its 6-OMe- and 7-OMe- derivatives **73** and **74** (Origitano and Collins 1980), whilst purified COMT from rat liver methylated 6,7-dihydroxy-TIQ at both phenolic groups (Creveling et al. 1972).

O-Methylations in isoquinoline derivatives by plants and cell cultures are discussed in Chapter 3. Parallel to experiments reported there, we investigated the metabolism of the salsolinol (**72**)-derivatives isosalsoline (**73**), salsoline (**74**), and *N*-methylisosalsoline (**75**) which occur in Papaveraceae and Fumariaceae plants (Iwasa et al. 1991). Thus we used calli of *Corydalis ochotensis* var. *raddeana, C. ophiocarpa* (Fumariaceae) and *Macleaya cordata* (Papaveraceae). Non-incubated calli of *C. ochotensis* var. *raddeana* contain isosalsoline (**73**), salsoline (**74**), and N-methylisosalsoline (**75**), indicating that O- and N-methylation normally occur within the biosynthesis of these small TIQs.

When (non-labelled, see below) racemic salsolinol (**72**) was administered to those calli cultures *via* the agar medium, the O-methylated derivatives isosalsoline (**73)** and *N*-methylisosalsoline (**75**) were obtained. Due to insufficient quantity of material for ^{1}H NMR spectra, a further metabolite was regarded to be salsoline (**74**). Later on this assumption was corrected by LC-API-MS which revealed this compound to be salsolidine (**77**) (Iwasa et al. 1992). In comparison with a "blank" experiment (no **72** added), however, the quantities of **73** and **75** are markedly increased, indicating **72** to be their precursor. Compound **73** was (–)-rotating, cpd. **75** rotated (+). In order to get unequivocal results, labelled salsolinol **76** (99% D_4; C-1-D; C-1-CD_3, prepared from dopamine plus CD_3-CDO at pH 4.5) was administered to these calli. Deuterated **73** and **74** were formed in a ratio of approximately 5:1. These derivatives showed 77% D_4, the derivative **75** also only 78% D_4. These diminutions are due to dilution by the corresponding non-deuterated alkaloids (see above). – When labelled **73** (77% D_4) so obtained was used as a precursor, its N-methyl derivative **75** arose as expected, but the deuterium content in **75** as well as in recovered **73** was decreased to 61% in **75** and to 66% in **73**. Also here, the deuterated compounds were diluted with their non-deuterated congeners. It is remarkable that no N-methylation of the catecholamine salsolinol (**72**) occurs; this reaction obviously requires preceding O-methylation. The amount of metabolites separated by prep. TLC was too small for measuring optical activity. For this aspect see the subsequent paragraph. – The results of feeding experiments with **80** to *C. ophiocarpa* and to *M. cordata* parallel those of *C. ochotensis* var. *raddeana*. *Corydalis pallida var. tenuis, C. incisa, M. cordata* (intact plants and calli), *C. ochotensis* var. *raddeana,* and *C. ophiocarpa* (calli) converted racemic [1-D, 1-CD_3]-6,7-dihydroxy-1-methyl-TIQ **76** to the deuterated, optically active metabolites (–)-isosalsoline (**73**; C-6-O-methylation), its (+)-rotating N-methyl derivative **75** and traces of salsolidine (**77**; twofold O-methylation). This was the first report on a bioconversion of rac. salsolinol (**72**) into optically active TIQs in animals or plants. The compounds occur as mixtures of enantiomers of varying composition. According to Lundström (1983) (+)-salsoline (**74**) has (*R*) configuration, and (–)-salsolidine (**77**) is (*S*) configurated. Thus we assume that (–)-**73** predominantly has (*S*) configuration whilst (+)-N-methylisosalsoline (**75**) is (*R*)-configurated. We could not determine the enantiomeric excess due to lack of material.

In another context, Strolin Benedetti et al. (1989) and Makino et al. (1990) have examined the varying rations of (*R*)- and (*S*)-salsolinol (**72**) in mammalian tissue and foods. They explained this fact by assuming dehydrogenation of **72** to the corresponding 1,2-dehydrosalsolinol, thus eliminating the centre of chirality, followed by asymmetric reduction.

This dehydrogenation process has been described by Bembenek et al. (1990). If this assumption is correct, our examination of the metabolites, generated by incubation with 1-D/1-CD_3 materials, should lose 1-D by dehydrogenation, and subsequent hydrogenation should result in a 1-H/1-CD_3-increment. In LC/APCI-MS, however, there were no $[M+H]^+$-ions of isosalsoline-D_3 (**73-D_3**) and N-methylisosalsoline-D_3 (**75-D_3**). The deviating results may be due to the difference between plants and mammalian tissues. Thus we conclude that stereoselective methylations of C-6-OH and NH occur. A preferential N-methylation of (*R*)-isosalsoline (**73**) can explain the (remaining) (*S*)-isosalsoline and the prevailing of (*R*)-*N*-methylisosalsoline (**75**). These methylations are the object of the next paragraph.

Thus we have compared literature results concerning O-methylation of dopamine (Iwasa et al. 1993b) in mammals with bioconversions of dopamine by *Corydalis pallida* var. *tenuis, C. ochotensis* var. *raddeana, C. incisa* (Fumariaceae), *Macleaya cordata* (Papaveraceae), and *Cynanchum vincetoxicum* (Asclepiadaceae), because phenolic phenylpropane increments are involved in the biosynthesis of the phenanthroindolizidine alkaloids of this plant.

According to Origitano et al. (1980) 7-O-methylated salsolinol, *id est* salsoline (**74**) occurs in heart and brain of rats, while 6- and 7-O-methylations were produced *in vitro* by rat liver. In contrast to these results in a mammal, we have shown, that salsolinol (**72**) was methylated by enzymes of Papaveraceae or Fumariaceae at C-6-OH, producing **73,** and to a low extend to the 6,7-dimethoxy-derivative **77** (salsolidine) without N-methylation. – Therefore, the main topic of our experiments

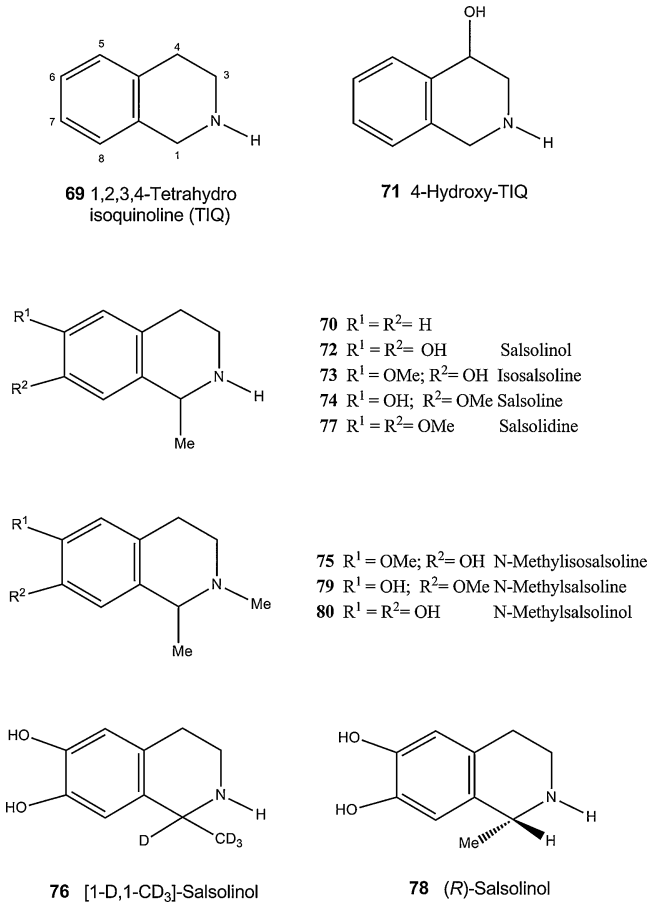
was O-methylation in the Fumariaceae and Papaveraceae plants mentioned above. Due to biochemical relations, dopamine-O-methylation was also investigated in *Cynanchum vincetoxicum* (Asclepiadaceae), because the phenanthroindolizidine alkaloids of this plant are biosynthesized *inter alia* from dopamine and tyramine, respectively (Wiegrebe 1972; Bick and Sinchai 1981). Therefore, calli of *C. pallida* were grown on an agar medium containing D_3C-CDO, dopamine and D,L-methionine as a source of methyl groups. Processing of calli and medium (Iwasa et al. 1987), followed by LC-APCI-MS as mentioned above, revealed the presence of D_4-isosalsoline (**73-D$_4$**) and a small amount of salsolidine (**77-D$_4$**; twofold O-methylation). ^{1}H - NMR spectra indicated a D-content > 90%. Parallel to *C. pallida,* in *C. ochotensis* var. *raddeana, C. incisa,* and *M. cordata*, deuterated salsolinol (**72**), isosalsoline (**73**), *N*-methylisosalsoline (**75**), and salsolidine (**77**) were obtained. These results show that dopamine is converted to salsolinol (**72**), which is further metabolized as indicated.

In the context of alcoholism, perdeuteriated ethanol was substituted for perdeuteuriated acetaldehyde as a precursor. Also here salsolinol (**72**), isosalsoline (**73**), *N*-methylisosalsoline (**75**), and salsolidine (**77**) were obtained in tetradeuterated form. Some dopamine was converted to 2-(4-hydroxy-3-methoxyphenyl)ethylamine (3-methoxytyramine) in *C. pallida* and *Cyn. vincetoxicum,* but this amine was scarcely converted to the corresponding TIQs **73** and **75**.

In subsequent experiments, O-methylation was thoroughly investigated. Thus, L-[Me-D_3]methionine or [Me_3-D_9]choline, resp., were administered to tissue cultures of *C. pallida* var. *tenuis* or *Cyn. vincetoxicum* because choline had been isolated from *Corydalis* plants (Tani et al. 1975a). O-Me- and N-Me-TIQs were generated in a remarkable higher amount after feeding of deuterated methionine as compared with choline as an incubator. Thus, it was evident, that SAM (S-adenosyl-L-methionine) is the main source of methyl groups. – In cultured cells of *Cyn. vincetoxicum* 3-methoxytyramin was generated besides the catecholamine salsolinol (**72**) which, however, was not methylated at the O-atoms. As a result of these methylation-aligned experiments, we concluded that the methylating enzyme is not COMT. This might be the reason why different O-methylations occur *in vitro* and *in vivo* in animals and plants. – Pyrogallol is a known inhibitor of COMT (Baldessarini and Chase 1972). Thus we studied the influence of pyrogallol on O-methylating enzymes in *C. pallida* var. *tenuis* and *Cyn. vincetoxicum* (Iwasa et al. 1995c). In order to get clear-cut results, we used D-labelled substrates. As a result, the formation of 7-hydroxy-6-methoxy-1-methyl-tetrahydroisoquinoline (**73**, isosalsoline) from the catecholamine salsolinol (**72**) is diminished by pyrogallol in calli of *C. pallida* var. *tenuis*, while O-methylation of dopamine to 3-methoxytyramine is not. These results are qualitatively similar to those obtained in intact plants and point towards O-methylating enzymes which differentiate between C-6-OH of salsolinol (**72**) and C-3-OH of dopamine in *C. pallida* var. *tenuis.*

While in this species enzymatic efficacies of intact plant and calli are similar, in *Cyn. vincetoxicum,* however, pyrogallol reduced the formation of 3-methoxytyramin from dopamine in plants, but not in their calli. Also O-methylation of salsolinol (**72**) in *Cyn. vincetoxicum* differs from that in *C. pallida* var. *tenuis*. In *Cyn. vincetoxicum* both O-methylated derivatives of salsolinol (**72**), that is to say isosalsoline (**73**) and salsoline (**74**), are generated in plants and in callus tissues. Most probably, the O-methylating enzymes for C-6-OH and C-7-OH are different. – In all these experiments metabolites come up in deuterated form as expected from the experimental conditions.

In the brain of humans, (*R*)-salsolinol (**78**) is enantioselectively synthesized from dopamine and acetaldehyde. Its



Scheme 10: Simple tetrahydroisoquinolines

N-methylated follow-up product (*R*)-*N*-methylsalsolinol (*R*-**80**) induces parkinsonism (Naoi et al. 1996; Maruyama et al. 1997). This N-methylation of salsolinol (**72**) itself had not been observed in plants or tissue cultures of *Corydalis* species. N-Methylation only occurs after preceding O-methylation, as shown by our feeding experiments (Iwasa et al. 1991, 1992). Thus these findings drew our special attention to this difference between mammalian and plant tissues (Iwasa 2004).

In order to get reference 1,2,3,4-TIQs, carnegine (6,7-dimethoxy-1,2-dimethyl-TIQ) was heated with HBr to give a mixture of phenolic TIQs which were identified by LC-APCI-MS and LC-NMR. As an extension of the analytical techniques used before, the positions of O-methylation were determined by stopped-flow NOEs. Thus, the data of the *mono*-O-methylated TIQs, *N*-methylsalsoline (**79**), of its regioisomer *N*-methylisosalsoline (**75**), of carnegine, and of 6,7-dihydroxy-1,2-dimethyl-TIQ (**80**; *N*-methylsalsolinol) could be recorded. Racemic [1-D,1-CD_3]salsolinol (**76**) was prepared from dopamine and D_3C-CDO (see above). This potential precursor was fed to cultured cells of *C. ochotensis* var. *raddeana* and other *Corydalis* species.

O-Methylation generated (deuterated) 7-hydroxy-6-methoxy-1-methyl-TIQ (**73-D$_4$**) and its (deuterated) N-methyl derivative **75-D$_4$**.Twofold O-methylation occurred also, leading to the sec. amine **77-D$_4$**. – In *C. platycarpa* the same metabolites of salsolinol (**72**) were biosynthesized. In the feeding experiments of *C. ochotensis* as well as *C. platycarpa* no N-methylation occurred without preceding O-methylation. This is in accordance with preceding results of our group.

When dopamine, D_3C-CDO and *S*-adenosyl-L-methionine, precursors of salsolinol (**72**) and of its O- and N-methyl groups, were administered to cell cultures of *C. platycarpa*, a mix-

ture of tetradeuterated salsolinol (**76**) and its non-deuterated congener **72** arose. Again, **73-D4** (isosalsoline) and 7-hydroxy-6-methoxy-1,2-dimethyl-TIQ (**75-D$_4$**; N-methylisosalsoline) in non-deuterated and deuterated form were produced, but no N-methylsalsolinol (**80**) was found although a supporting methyl donor had been administered. These results show a profound difference between N-methylating enzymes in plants and in mammalian brains, resp.

To our feeling, we have settled the issues of O- and N-methylated TIQs in *Corydalis* species and *Cyn. vincetoxicum* as far as methylated products are concerned. Deeper insight might be obtained by studying the purified enzymes involved, but here we are not competent.

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