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The biosynthesis of papaverine proceeds *via* (*S*)-reticuline ☆

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ABSTRACT

Papaverine is one of the earliest opium alkaloids for which a biosynthetic hypothesis was developed on theoretical grounds. Norlaudanosoline (=tetrahydropapaveroline) was claimed as the immediate precursor alkaloid for a multitude of nitrogen containing plant metabolites. This tetrahydroxylated compound was proposed to be fully *O*-methylated. The resulting tetrahydropapaverine should then aromatize to papaverine. In view of experimental data, this pathway has to be revised. Precursor administration to 8-day-old seedlings of *Papaver* followed by direct examination of the metabolic fate of the stable-isotope-labeled precursors in the total plant extract, without further purification of the metabolites, led to elucidation of the papaverine pathway *in vivo*. The central and earliest benzylisoquinoline alkaloid is not the tetraoxygenated norlaudanosoline, but instead the trihydroxylated norcoclaurine that is further converted into (*S*)-reticuline, the established precursor for poppy alkaloids. The papaverine pathway is opened by the methylation of (*S*)-reticuline to generate (*S*)-laudanine. A second methylation at the 3′ position of laudanine leads to laudanosine, both known alkaloids from the opium poppy. Subsequent *N*-demethylation of laudanosine yields the known precursor of papaverine: tetrahydropapaverine. Inspection of the subsequent aromatization reaction established the presence of an intermediate, 1,2-dihydropapaverine, which has been characterized. The final step to papaverine is dehydrogenation of the 1,2-bond, yielding the target compound papaverine. We conclusively show herein that the previously claimed norreticuline does not play a role in the biosynthesis of papaverine.

1. Introduction

Papaverine (**1**) is a benzylisoquinoline alkaloid (Fig. 1) that was discovered by Merck (1848) as a minor (ca. 1%) component in the latex of the opium poppy (*Papaver somniferum* L.). It occurs also in other members of the genus *Papaver*. Due to selection for morphinans in cultivars of *P. somniferum*, papaverine (**1**) is either a very minor alkaloid or totally absent. The pharmacological effect of papaverine (**1**) is on cerebral blood flow; it possesses spasmolytic and vasodilatory activity. The isolated natural product drug was eventually replaced by the synthetic drug for commercial use after development of an efficient chemical synthesis (Taylor and Martin, 1974). Papaverine (**1**) was also used to correct male impotence, but has now been substituted by the more effective Sildenafil.

Papaverine (**1**) is an interesting target for biosynthetic studies. This molecule is fully *O*-methylated on the hydroxyl groups in positions 6, 7, 3′, and 4′; it lacks, however, an *N*-methyl group and its B-ring is aromatic. Winterstein and Trier in 1910 postulated that plant-derived tetrahydrobenzylisoquinoline alkaloids were derived from the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde to form norlaudanosoline (**2**) (Fig. 2). Following this early biogenetic prediction, Battersby and co-workers conducted experiments that seemingly verified and extended the Winterstein and Trier hypothesis (Battersby and Binks, 1960; Battersby, 1963; Battersby et al., 1964). Application of position-specific labeled tyramine as precursor and tedious specific degradation of the labeled papaverine (**1**), established that this alkaloid was indeed formed from two C_6–C_2 units derived from dopamine and 3,4-dihydroxyphenylacetaldehyde (Battersby, 1963). Furthermore, it was postulated that papaverine (**1**) is formed: (1) from norlaudanosoline (**2**) (Battersby et al., 1965), (2) from (*S*)-norreticuline (**4**) (Battersby, 1963) as well as several other alkaloids from the nor-series such as norlaudanine and tetrahydropapaverine (possessing no *N*-methyl group). The latter, however, was claimed to be not significantly incorporated into papaverine (**1**) due to the lack of

☆ This paper is dedicated to Prof. Rolf Huisgen on the occasion of his 90th birthday.

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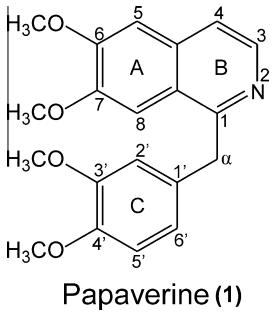

Fig. 1. Papaverine (**1**) and its three aromatic rings A, B and C.

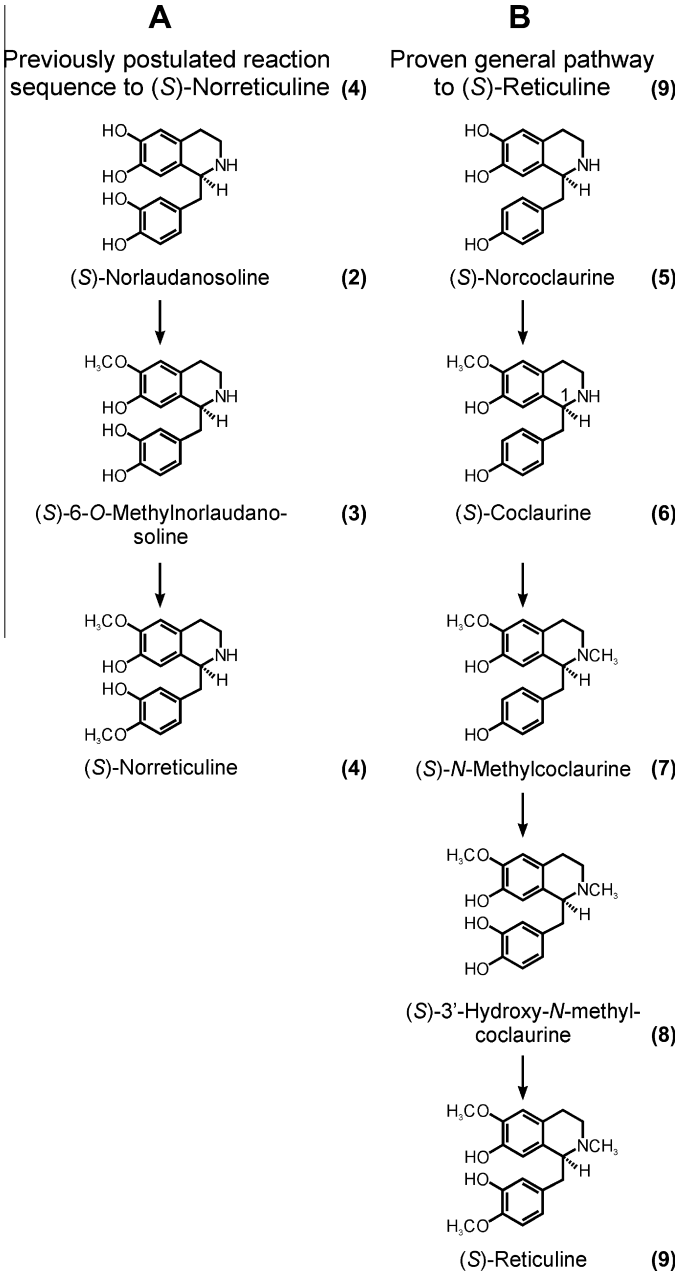

Fig. 2. Postulated and verified sequence of reactions for the formation of benzylisoquinoline alkaloids. (A) Previously postulated sequence of reactions for the biosynthesis of (*S*)-norreticuline (**4**), as precursor to papaverine (**1**); (B) general pathway and sequence of reactions verified for the biosynthesis of benzylisoquinoline alkaloids (*via* (*S*)-reticuline (**9**)).

an unmethylated phenolic hydroxyl group (Battersby et al., 1964). Independently, the group of Brochmann-Hanssen (Brochmann-Hanssen et al., 1971, 1975, 1980) extended these suggestions and claimed that norreticuline (**4**), norlaudanine and norlaudanosine were easily incorporated into papaverine (**1**), while reticuline (**9**) was not (Uprety et al., 1975). This proposal received support by the recent claim that a SAM-dependent *O*-methyltransferase exists that accepts norreticuline (**4**) as the sole substrate (Pienkny et al., 2009). Our recent findings, however, are at variance to the above claims. Norlaudanosoline (**2**), norreticuline (**4**), norlaudanine or norlaudanosine have never been found in higher plants as natural products despite meticulous phytochemical work. Isotope dilution analysis showed that norlaudanosoline (**2**) and norreticuline (**4**) were not detected during isoquinoline alkaloid synthesis (Stadler and Zenk, 1990). Furthermore, the trihydroxylated (*S*)-norcoclaurine (**5**) is the central precursor of benzylisoquinoline alkaloids and not the long postulated (*S*)-norlaudanosoline (**2**) (Stadler et al., 1987; Stadler and Zenk, 1990; Frenzel and Zenk, 1990a). The sequence of enzymatically catalyzed methylation and oxidation steps of (*S*)-norcoclaurine (**5**) excludes the existence of (*S*)-norreticuline (**4**), the postulated precursor of papaverine (**1**) (Fig. 2). The sequence is 6-*O*-methylation, *N*-methylation, hydroxylation of 3′ position and 4′-*O*-methylation.

Hence the above-described hypothesis of a pathway for the formation of papaverine (Fig. 2A), does not have experimental support. The incorporation of potential synthetic precursors such as norreticuline (**4**), and even their correct position-specific incorporation into papaverine (**1**), may be attributed to the promiscuous enzyme activities such as are found in the plant kingdom. All of these inconsistencies in the hitherto published literature and textbooks justified revisiting the biosynthesis of papaverine (**1**). We approached a solution to this problem by using high resolution mass spectrometry together with stable-isotope-labeled potential precursors and 8-day-old seedlings of an opium poppy hybrid that accumulate sufficient amounts of papaverine (**1**) for trace analysis of intermediates as well as end products.

2. Results and discussion

To begin our investigation, we analyzed the pertinent alkaloids in the methanolic extract of 8-day-old poppy hybrid seedlings *via* high resolution LC–MS. The alkaloid pattern and the molecular characteristics are shown in Table 1.

As can be noted from the retention times in Table 1, the individual alkaloids are clearly separated from each other by high resolution LC–MS, which is a prerequisite for using isotopically labeled compounds. No prediction can be made at this stage regarding the stereochemistry of these alkaloids. Thebaine (**10**) was used as an indicator for the presence of the morphine pathway. In order to establish the origin of the aromatic ring systems in the alkaloids

Table 1
High resolution LC–MS/MS data and quantified amounts of pertinent alkaloids from poppy hybrid seedlings.

Compound	$[M+H]^+$ [*m*/*z*] (experimental)	$[M+H]^+$ [*m*/*z*] (theoretical)	Retention time (min)	Amount (nmol/g dry weight)
Reticuline (**9**)	330.17011	330.16998	7.78	2.53
Laudanine (**11**)	344.18542	344.18563	12.74	0.06
Laudanosine (**12**)	358.20112	358.20128	14.79	0.06
Tetrahydropapaverine (**13**)	344.18546	344.18563	11.59	0.40
Papaverine (**1**)	340.15417	340.15433	16.76	0.98
Thebaine (**10**)	312.15929	312.15942	12.61	3.71

of interest, we applied the chemically more stable 6-*O*-methylated derivative, [1-^{13}C]-(*S*)-coclaurine (**6**) (Fig. 2B), to the poppy seedlings. As a result of the precursor administration experiment, we found 15.08 nmol labeled and 41.86 nmol unlabeled thebaine (**10**) resulting in a ratio of 0.265 for the quotient of labeled to unlabeled alkaloid. Calculation in an analogous manner for papaverine (**1**) resulted in a quotient of 0.017. Based on dry weight, this calculation corresponds to 0.04% papaverine (**1**) and 0.4% thebaine (**10**). The conclusion from the result of this precursor administration experiment is that the universal precursor (*S*)-coclaurine (**6**) is incorporated into both papaverine (**1**) and thebaine (**10**), but it cannot be excluded that coclaurine (**6**) was processed further without an *N*-methyl group to furnish papaverine (**1**).

As quoted above (Stadler and Zenk, 1990), norreticuline (**4**) has never been found in plants, therefore we applied [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) to the poppy hybrid. The reason for the use of the double labeled compound was that the [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) ought to be incorporated into papaverine (**1**) while the *N*-methyl group should be lost during the transition to the target compound. (*S*)-Reticuline (**9**) is one of the central precursors for a multitude of benzylisoquinoline alkaloids retaining the *N*-methyl group such as morphinans, aporphines, protoberberines and benzo[*c*]phenanthridines (Kutchan, 1998). The substitution pattern of the phenolic groups means that a biosynthetic relationship to papaverine (**1**) could be envisaged, if it is assumed that *N*-demethylation is necessary in the course of the pathway to papaverine (**1**). To test this possibility, we observed the flow of [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) into the hypothetical biosynthetic pathway of papaverine (**1**).

Interestingly, the majority of labeled [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) was transformed into thebaine (**10**) (4.35 nmol/g dry weight) while the incorporation into (*S*)-laudanine (**11**) and (*S*)-laudanosine (**12**), with the two labeled positions intact, was much less (0.1 nmol/g dry weight, Table 2). This difference was due to the fact that the metabolic route to thebaine (**10**) is the major pathway in the used poppy cross, while the one to papaverine (**1**) is only a side pathway. The detection of labeled laudanine (**11**) and laudanosine (**12**) was not sufficient to demonstrate that both alkaloids were precursors of papaverine (**1**) rather than simply natural products occurring in the plant. However, the same reticuline (**9**) administration also resulted in single labeled tetrahydropapaverine (**13**) and papaverine (**1**) at the original 1-^{13}C position, while the *N*-methyl group was lost.

This demonstrates that (*S*)-reticuline (**9**) is the precursor to tetrahydropapaverine (**13**) and papaverine (**1**). The question remains, however, which pathway (*S*)-reticuline (**9**) takes on its way to these two *N*-demethylated compounds. As a control, we analyzed the incorporation of the doubly-labeled reticuline (**9**) into thebaine (**10**), which gave an incorporation quotient of 0.54. This demonstrated the dominance of the morphine pathway over the papaverine pathway (Table 2).

Since laudanine (**11**), the *O*-trimethylated, *N*-methylated benzylisoquinoline alkaloid, occurs in the opium poppy, and a recombinant SAM-dependent enzyme is known that methylates (*S*)-reticuline (**9**) at the 7 position to yield laudanine (**11**) (Ounaroon et al., 2003), it was tempting to test whether this precursor was incorporated into papaverine (**1**). (*S*)-Laudanine (**11**) was synthesized by methylating (D_3-methyliodide) (*S*)-reticuline *N*-oxide with subsequent deprotection (H_2SO_3) (Phillipson et al., 1976; Lu et al., 1987). This method yielded both [7-D_3]-laudanine (**11**), as well as [3′,7-D_6]-laudanosine (**12**), the former having +3 mass units due to the deuterium label while the latter had +6 mass units. The alkaloids were separated by TLC and their constitution verified by high resolution LC–MS (Fig. 3A and B). [7-D_3]-laudanine (**11**) and [3′,7-D_6]-laudanosine (**12**) were administered individually to poppy seedlings, as described in Section 4. The result of this application experiment showed that [7-D_3]-laudanine (**11**) was incorporated into papaverine (**1**) to 53.6% of the total papaverine (**1**) present, while [3′,7-D_6]-laudanosine (**12**) was incorporated to 66.7% of the total target compound (Fig. 3C and D). This experiment and the high incorporations show without any doubt that the *N*-methylated alkaloids are excellent precursors and that in the biochemical event, the *N*-methyl group is removed and the resulting alkaloid is dehydrogenated to yield papaverine.

Having established the precursor function of laudanine (**11**) by efficient incorporation into papaverine (**1**), we set out to explore the missing biochemical steps between laudanine (**11**) and papaverine (**1**). Precursor administration experiments using [7-D_3]-laudanine (**11**) showed in the full scan the following four labeled intermediates calculated in percentage based on the total amount (labeled and unlabeled) of each alkaloid present in the seedling: laudanosine (**12**) (98.6%), tetrahydropapaverine (**13**) (missing the *N*-methyl group) (96.4%), an unknown metabolite (99.2%) and papaverine (**1**) as shown before (53.6%), the obvious end product. Administering [7-^{14}C]-labeled laudanine (**11**) to poppy seedlings, subjecting the total extract (without fractionation) to 2D TLC followed by exposure on a phosphorimager screen revealed only the four radioactive products, laudanosine (**12**), tetrahydropapaverine (**13**), the unknown metabolite and papaverine (**1**), and no additional metabolites (data not shown).

The occurrence of stable-isotope-labeled tetrahydropapaverine (**13**) demonstrates, that in the poppy plant, the fully methylated alkaloid laudanosine (**12**) is subjected to demethylation. This is the missing step between the *N*-methylated tetrahydrobenzylisoquinoline alkaloid and the *N*-demethylated papaverine skeleton. The intermediate is (*S*)-tetrahydropapaverine (**13**), a compound first postulated in papaverine (**1**) biosynthesis (Brochmann-Hanssen et al., 1971) after it had been found as a natural product isolated from *P. somniferum*. This postulate was verified by Brochmann-Hanssen et al. (1975) who synthesized (−)-tetrahydropapaverine (**13**) that was found to be incorporated into papaverine (**1**) with an, at that time, extremely high incorporation rate in the poppy plant. However, the opinion was that (*S*)-norreticuline (**4**) and norlaudanosoline (**2**) were the precursors of norlaudanine, norlaudanosine, with the biosynthetic grid ending with tetrahydropapaverine (**13**) and papaverine (**1**) (Battersby, 1963;

Table 2
High resolution LC–MS/MS data and quantified amounts of pertinent alkaloids from the [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) administration experiment. Note: Administration of doubly-labeled [1-^{13}C, *N*-^{13}CH$_3$]-(*S*)-reticuline (**9**) to poppy plants leads to the formation of doubly-labeled (1-^{13}C, *N*-^{13}CH$_3$) laudanine and laudanosine, but singly-labeled (1-^{13}C) tetrahydropapaverine (**1**) and singly-labeled (1-^{13}C) papaverine (**1**) while thebaine is doubly-labeled (1-^{13}C, *N*-^{13}CH$_3$) demonstrating the branch point.

Compound	$[M+H]^+$ [*m*/*z*] (experimental)	$[M+H]^+$ [*m*/*z*] (theoretical)	Retention time (min)	Amount (nmol/g dry weight)
[1-^{13}C, *N*-^{13}CH$_3$]-Reticuline (**9**)	332.17652	332.17669	7.78	95.30
[1-^{13}C, *N*-^{13}CH$_3$]-Laudanine (**11**)	346.19247	346.19234	12.74	0.10
[1-^{13}C, *N*-^{13}CH$_3$]-Laudanosine (**12**)	360.20786	360.20799	14.79	0.02
[1-^{13}C]-Tetrahydropapaverine (**13**)	345.18873	345.18899	11.59	0.01
[1-^{13}C]-Papaverine (**1**)	341.15751	341.15769	16.77	0.01
[1-^{13}C, *N*-^{13}CH$_3$]-Thebaine (**10**)	314.16595	314.16613	12.61	4.35

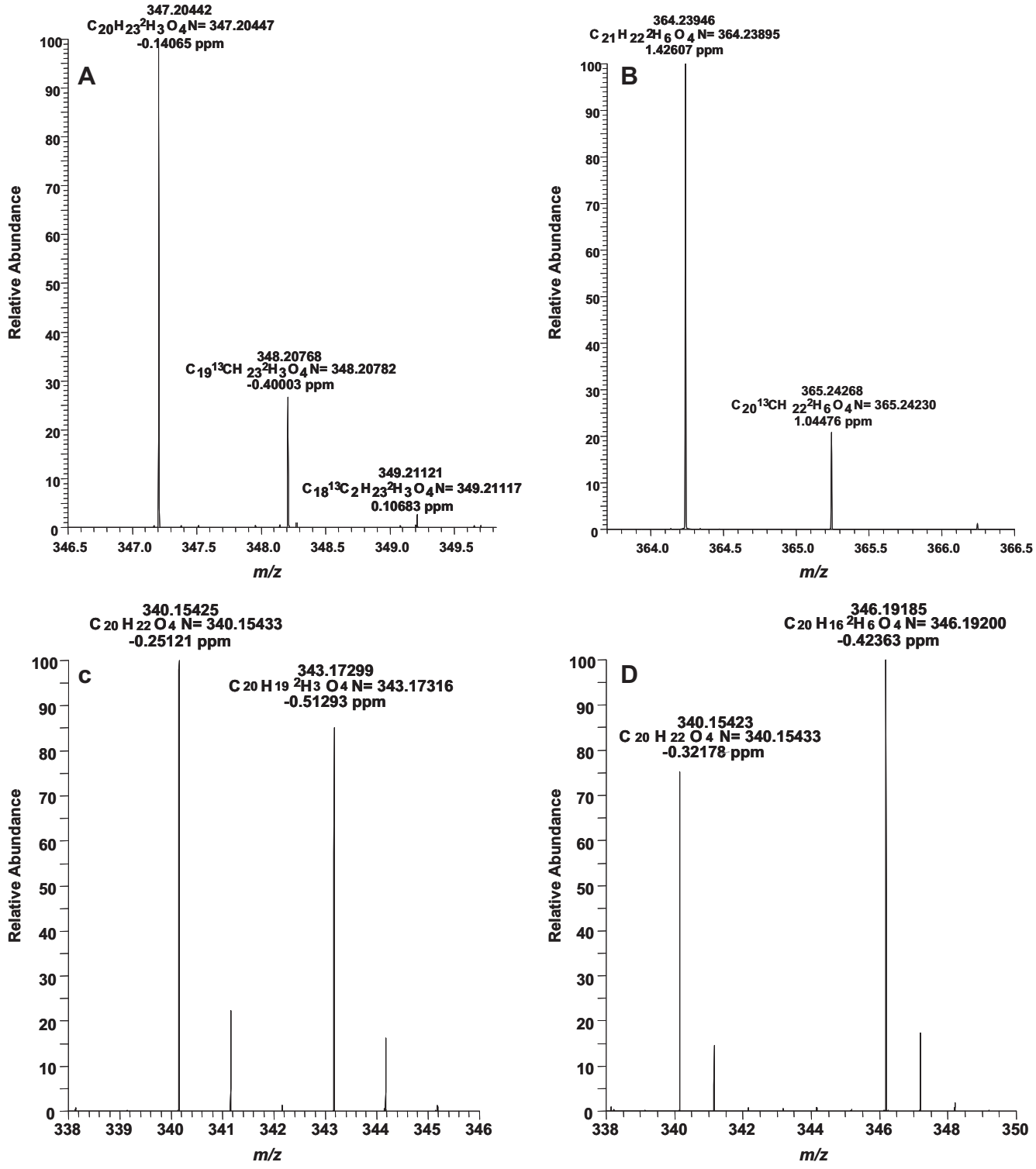

Fig. 3. High resolution mass spectra. (A) isotopically synthesized [7-D_3]-laudanine (**11**); (B) isotopically synthesized [3′,7-D_6]-laudanosine (**12**); (C) biosynthetically obtained unlabeled and [7-D_3]-papaverine (**1**); (D) biosynthetically obtained unlabeled and [3′,7-D_6]-papaverine (**1**).

Brochmann-Hanssen et al., 1971; Uprety et al., 1975) but, as mentioned before, Brochmann-Hanssen and associates never found norreticuline (**4**), norlaudanine or norlaudanosine.

We investigated further the precursor role of tetrahydropapaverine (**13**) in the biosynthesis of papaverine (**1**). The administration of deuterated (*R*,*S*)-tetrahydropapaverine (**13**) was carried out as usual. The plants were extracted with methanol and subjected to high resolution mass spectrometry. Quantitative mass spectrometric analysis revealed a quotient of 0.81 for the incorporation of deuterium-labeled (*R*,*S*)-tetrahydropapaverine (**13**) (isotopic distribution < 0.1% [$^2H_0$]/ < 0.1% [$^2H_1$]/ < 0.1% [$^2H_2$]/ < 0.1% [$^2H_3$]/3% [$^2H_4$]/21.5% [$^2H_5$]/39% [$^2H_6$]/29.9% [$^2H_7$]/5.5% [$^2H_8$]/1% [$^2H_9$]) into papaverine (**1**) and an isotopic distribution for deuterium-labeled papaverine (**1**) of 11.8% [$^2H_0$]/2.9% [$^2H_1$]/12% [$^2H_2$]/

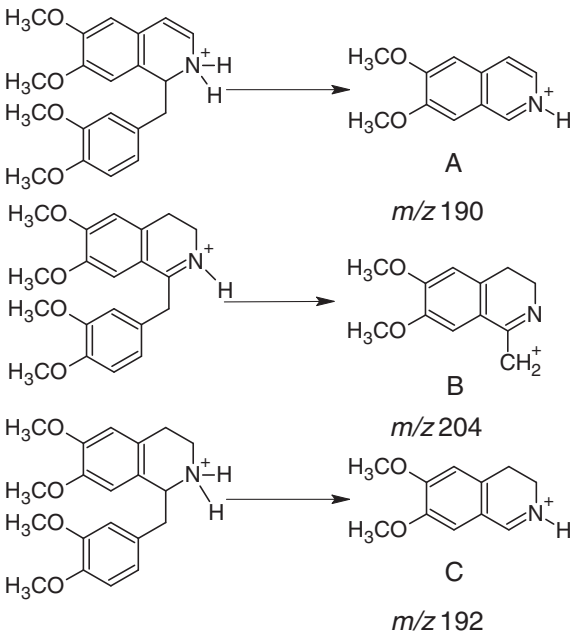

Fig. 4. Two possible structures for the biosynthetic intermediate between tetrahydropapaverine (**13**) and papaverine (**1**) and mass fragmentation of tetrahydropapaverine (**13**). (A) 1,2-Dihydropapaverine (**14**), the naturally occurring compound, and its product ion *m*/*z* 190; (B) 3,4-Hydropapaverine and its predicted product ion *m*/*z* 204; (C) Tetrahydropapaverine (**13**) and its product ion *m*/*z* 192.

32.2% [$^2H_3$]/30% [$^2H_4$]/11% [$^2H_5$]/0.1% [$^2H_6$]. This high incorporation rate is explained by the immediate precursorship of tetrahydropapaverine (**13**) to papaverine (**1**). The loss of three deuterium atoms during the aromatization process is reflected by the isotopic distribution pattern of labeled tetrahydropapaverine (**13**) and labeled papaverine (**1**) with the major peaks changing from D_6 and D_7 to D_3 and D_4, respectively. The precursor administration experiment with deuterated (*R*,*S*)-tetrahydropapaverine (**13**) established, in addition to labeled papaverine (**1**), the presence of an intermediate *m*/*z* 342.16992 ($C_{20}H_{24}O_4N$) with an isotopic distribution of <0.1% [$^2H_0$]/ < 0.1% [$^2H_1$]/3% [$^2H_2$]/1% [$^2H_3$]/18% [$^2H_4$]/32% [$^2H_5$]/24% [$^2H_6$]/21% [$^2H_7$]/1% [$^2H_8$]. The intermediate was identified as labeled 1,2-dihydropapaverine (**14**) showing an incorporation quotient of 0.28 and, for *m*/*z* 347.20137 carrying five deuterium atoms, a fragment ion of *m*/*z* 195.11800 ($C_{11}H_7{}^2H_5O_2N$). The formation of labeled 1,2-dihydropapaverine (**14**) from labeled tetrahydropapaverine (**13**) goes along with the loss of two deuterium atoms. Indeed, the isotopic distribution patterns of both alkaloids established that a decrease in the number of D_6- and D_7-labeled isotopes by 15% and 8%, respectively, leads to an equivalent increase in the number of 1,2-dihydropapaverine (**14**) isotopes carrying four and five deuterium atoms. This experiment thus clearly demonstrated tetrahydropapaverine (**13**) as a substrate for the aromatization process in poppy plants.

This result was confirmed by a detailed analysis of the [D_6]-laudanosine (**12**) administration, which gave an additional peak between tetrahydropapaverine (**13**) and papaverine (**1**) in the mass chromatograms at 13.66 min with quasimolecular ions at *m*/*z* 342.16992 ($C_{20}H_{24}O_4N$) and 348.20760 ($C_{20}H_{18}{}^2H_6O_4N$) showing that an intermediate with two hydrogens less than tetrahydropapaverine (**13**) was formed. The MS/MS spectrum of the new compound showed fragments at *m*/*z* 190.08629 ($C_{11}H_{12}O_2N$) and 193.10512 ($C_{11}H_9{}^2H_3O_2N$), respectively. As discussed earlier (Battersby et al., 1977), the dehydrogenation of tetrahydropapaverine (**13**) to papaverine (**1**) may involve either a double bound formation between C_1 and N or between C_3 and C_4. The formation of the detected product ion *m*/*z* 190 (Fig. 4A) was in good agreement with the mass fragmentation of isoquinoline alkaloids shown by Wu and Huang (2006). Papaverine (**1**), containing a double bond between C_1 and N and between C_3 and C_4, gives the major R-CH_2^+ (R = isoquinoline) fragment *m*/*z* 202 (Wickens et al., 2006). A compound with a double bond located between C_1 and N would also give a R-CH_2^+ fragment, *m*/*z* 204 (Fig. 4B), due to its mesomeric stability. Because the dihydro-intermediate does not form a R-CH_2^+ fragment (Fig. 4A), it can be inferred that the additional double bond is located between C_3 and C_4. In addition, the MS/MS experiment of tetrahydropapaverine (**13**) showed a product ion with *m*/*z* 192, which supports the structure of a 1,2-dihydro derivative (**14**) (Fig. 4C).

For decades, norlaudanosoline (**2**) was assumed to be the first alkaloidal precursor for the major classes of plant-derived isoquinoline alkaloids (Winterstein and Trier, 1910; Robinson, 1917; Spenser, 1969). This hypothetical intermediate was seemingly verified, when it was shown that the expected labeled precursors were incorporated into papaverine (**1**), the target compound (Battersby and Harper, 1959, 1962; Battersby et al., 1964), despite some discrepancies in the labeling patterns (for review see Spenser, 1969) produced in poppy and other isoquinoline alkaloid accumulating plants. Extensive phytochemistry, precursor synthesis and experimentation by Brochmann-Hanssen and associates (Brochmann-Hanssen and Furuya, 1964; Brochmann-Hanssen et al., 1965, 1971, 1975) not only discovered the now recognized true precursors of papaverine (**1**), the opium poppy-derived laudanine (**11**) (laudanidine) and laudanosine (**12**) alkaloids, but found in labeled (*S*)-tetrahydropapaverine (**13**) an excellent precursor for papaverine (**1**), while (*R*)-tetrahydropapaverine is largely inactive. However, (*R*,*S*)-reticuline (**9**) was very poorly incorporated into papaverine (**1**), but most importantly the highest incorporation rates into papaverine (**1**) were achieved with norreticuline (**4**), which is missing the *N*-methyl group. These results led to the erroneous assumption that, as predicted by Winterstein and Trier (1910) and then experimentally shown by Battersby (1963), norlaudanosoline (**2**) and norreticuline (**4**) were precursors for papaverine (**1**). It was not yet known at that time that plant methyltransferases, and especially those from poppy, are promiscuous (Brochmann-Hanssen et al., 1980; Wat et al., 1986; Frenzel and Zenk, 1990b). Thus exogenously provided norlaudanosoline (**2**) and norreticuline (**4**) could easily be methylated at the nitrogen and phenolic groups by these methyltransferases. This promiscuity was demonstrated by us when poppy seedlings were administered [1-^{13}C]-(*S*)-norreticuline (**4**), which resulted in formation of singly-labeled (*S*)-reticuline (**9**) and singly-labeled thebaine (**10**), from which we found a ratio of 0.01 for the quotient of labeled to unlabeled compound. Supporting this is the fact that the unnatural alkaloid nororientaline is an excellent precursor to papaverine (**1**), since presence of the 7-*O*-methyl group prevents funneling into the thebaine (**10**) pathway (Brochmann-Hanssen et al., 1975).

With the knowledge of the biosynthesis of isoquinoline alkaloids, the use of alkaloid producing seedlings, methanolic total plant extracts and state-of-the-art mass spectrometry, we were able to decipher the biosynthesis of papaverine (**1**), one of the oldest targets of phytochemistry. The biosynthetic pathway as it stands now is shown in Fig. 5. None of the predicted previous intermediates with the exception of (*S*)-tetrahydropapaverine (**13**), is present in the new scheme depicted here.

3. Conclusion

By administering-heavy-isotope labeled potential precursors to seedlings of a poppy cross that produces both thebaine (**10**) and papaverine (**1**), and analyzing extracts by HPLC-high resolution

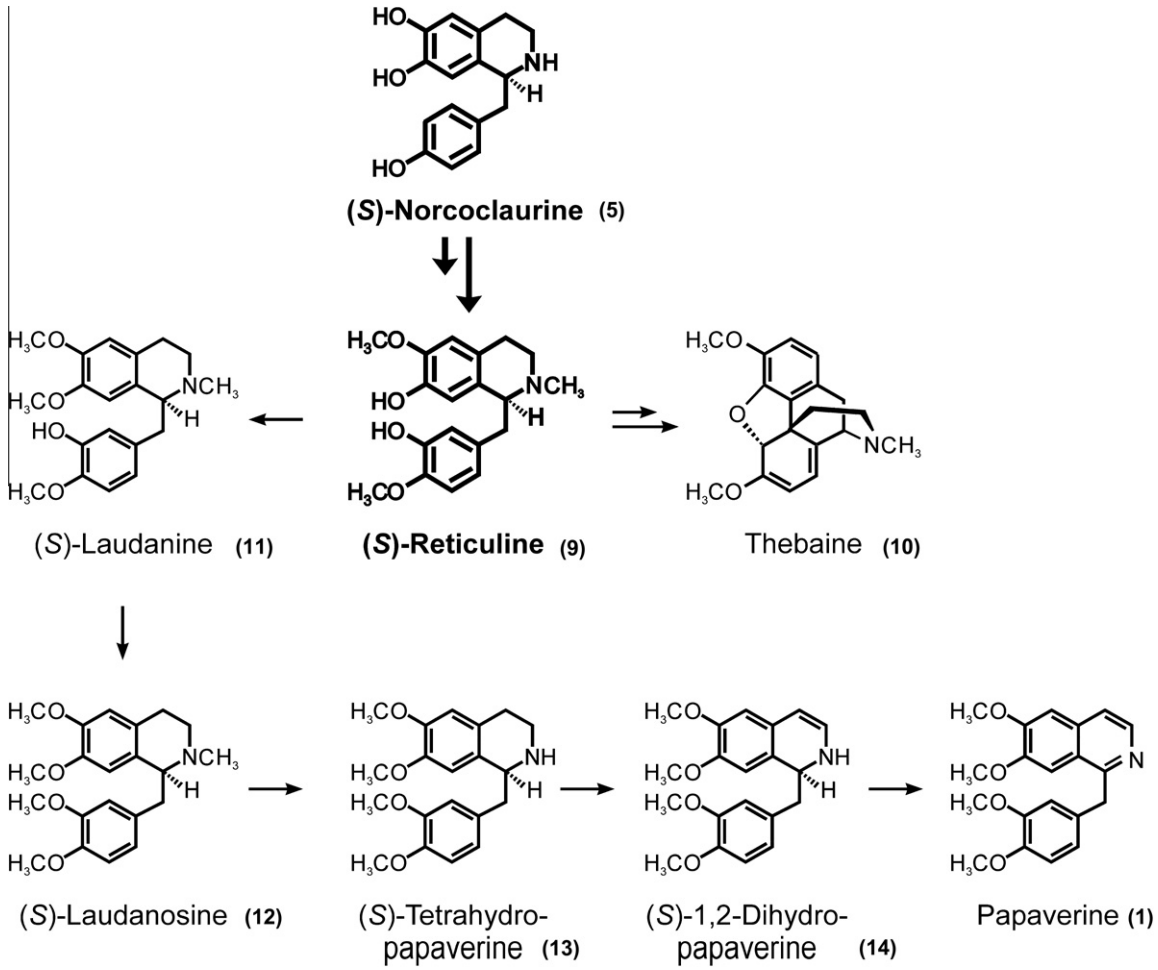

Fig. 5. New biosynthetic pathway to papaverine (**1**) in poppy plants. Shown in bold is the common pathway leading to both papaverine (**1**) and thebaine (**10**).

mass spectrometry, it was possible to resolve the controversial biosynthesis of papaverine (**1**) (Fig. 5). This methodology may be applied to other biosynthetic problems in the future to resolve similar types of questions in plant and animal systems. It is advisable to use a quotient of the incorporation of diverse unlabeled and position-labeled precursors. This procedure allows the study of a biosynthetic pathway using only small amounts of seedling tissue or plant cell suspension culture and omits tedious purification steps of the target compounds.

4. Experimental

4.1. Papaver

Hybrid seeds were produced by pollinating emasculated buds of *P. somniferum* with pollen of *P. somniferum* ssp. *setigerum*. Seeds of the F1 *P. somniferum* × *P. somniferum* ssp. *setigerum* cross were surface sterilized with hypochlorite. Twenty-five seeds were grown on filter paper in Petri dishes in a minimum quantity of Linsmaier Skoog mineral solution to allow uninhibited growth. The seedlings were grown in a 12-h day/night cycle under incandescent lamps at 20 °C. After a growth period of 4 days, the developing seedlings (25) were administered 350 μl of a sterile 0.5 mM solution of potential precursor which was taken up by the roots. The seedlings were supplied fresh precursor solution every 24 h for the following 3 days. On day 8, the seedlings were harvested and extracted with boiling MeOH–H_2O (20 ml, 1:4) (extracted dry weight 10.1 mg). The soluble extract was evaporated to dryness, taken up with MeOH (100 μl) and directly injected (10 μl) without fractionation onto the LC–MS instrument.

4.2. Mass spectrometry

High resolution mass spectra were obtained using an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA). The spectrometer was operated in the positive mode (1 spectrum s^{-1}; mass range: 50–1000) with a nominal mass resolving power of 60,000 at m/z 400 at a scan rate of 1 Hz using automatic gain control to provide high-accuracy mass measurements (⩽2 ppm deviation). The internal calibration standard bis-(2-ethylhexyl)-phthalate (m/z 391.28428) was used for the determination of elemental composition. The spectrometer was equipped with a Surveyor HPLC system (Thermo Scientific, USA) consisting of LC-Pump, UV detector (λ = 254 nm) and autosampler (injection volume 10 μl). Separation of samples was achieved by using a Machery–Nagel Nucleodor Gravity column (1.8 μm, 50 × 3 mm) combined with an associated guard column (4 × 3 mm). The mobile phase total flow was set to 0.4 ml/min with binary gradient elution, using solvents A (0.1% HCO_2H, 10 mM $(NH_4)OAc$) and B (0.1% HCO_2H in CH_3CN) (all v/v). The gradient started with 5% B for 4 min and was increased to 30% B over 20 min. Elution was continued for 10 min at 100% B followed by a 7-min equilibration with the starting condition. MS/MS spectra were obtained from the corresponding parent ions ($[M+H]^+$) with a collision energy of 35 V. The quantitation of the individual labeled compound was achieved by integration of the peak area in the extracted ion chromatogram with a mass range

of 2 ppm. All calibration graphs were linear ($R^2 > 0.99$) from 20 ng ml^{-1} up to a concentration of 20,000 ng ml^{-1}.

4.3. Chemical synthesis

4.3.1. [7-D_3]-laudanine (**11**) and [3′,7-D_6]-laudanosine (**12**)

m-Chloroperbenzoic acid (100 mg) dissolved in CH_2Cl_2 (5 ml) was added dropwise to a stirred solution (0°) of (*S*)-reticuline (**9**) (100 mg) in CH_2Cl_2 (15 ml) to yield (*S*)-reticuline *N*-oxide (66%) after 2.5 h (Phillipson et al., 1976; Lu et al., 1987). The (*S*)-reticuline *N*-oxide was purified *via* thin layer chromatography (TLC) on Polygram silica G/UV254 plates (Macherey–Nagel, layer: 0.2 mm silica gel with fluorescent indicator UV254) using the solvent system, acetone:H_2O:NH_4OH (80:15:5). The purified *N*-oxide was suspended in a mixture of EtOH (3 ml) and THF (9 ml) and reacted with D_3-methyliodide (10 equiv) and KOH (7.5 equiv) overnight at room temperature to methylate the *N*-oxide. After methylation, the reaction mixture was dried and dissolved in EtOH–H_2O (8 ml, 1:1). To remove the *N*-oxide protecting group, $NaHSO_3$ (400 mg) was added and stirred for 5 h. The reaction mixture was washed with CH_2Cl_2 and purified *via* the TLC system toluene:EtOAc:Et_2NH (7:2:1) to obtain [7-D_3]-laudanine (**11**) in 10% yield (Rf: 0.17) and [3′,7-D_6]-laudanosine (**12**) in 30% yield (Rf: 0.58). Several other compounds carrying deuterium label were noted on the chromatogram and clearly separated from each other, but their structures were not determined. Rechromatography was done on [7-D_3]-laudanine (**11**) using the TLC system xylene:2-butanone:2-propanol:Et_2NH (20:20:3:1). The isotopic distribution of d_3- and d_6-labeled alkaloids exhibited an isotopic purity >99.5%. [7-D_3]-Laudanine (**11**) and [3′,7-D_6]-laudanosine (**12**) were identified by comparison with unlabeled authentic standards, exhibiting the same chromatographic and spectroscopic data (Fig. 3A and B), except for their mass spectra, which show the calculated shift of their quasimolecular ion $[M+H]^+$ either +3 or +6, respectively (Fig. 6).

4.3.2. Deuterated tetrahydropapaverine (**13**)

Deuterated tetrahydropapaverine (**13**) was synthesized from papaverine (**1**) according to Taylor (1952) with slight modifications. To introduce a deuterium label at positions C_1, C_3 and C_4, papaverine (**1**) was refluxed in deuterium chloride and zinc dust. After 30 h of reflux, the reaction mixture was made alkaline and extracted with Et_2O (2 × 20 ml). The presence of tetrahydropapaverine (**13**) in the combined Et_2O phase was confirmed by TLC in the solvent system xylene:butanone:MeOH:Et_2NH (20:20:3:1). Et_2O was removed in vacuo, with the residue reconstituted in MeOH–CH_3CN (2:1). Deuterated tetrahydropapaverine (**13**) was further purified by HPLC (Merck Hitachi) using a Hibar Pre-Packed column RT250-25 (Merck, 7 μm, LiChrosorb RP-18). The mobile phase total flow was set to 8 ml/min with binary gradient elution using 0.1% CF_3CO_2H (solvent A) and CH_3CN (solvent B). The gradient started with 20% B and was increased to 50% B over 60 min. Elution was continued for 20 min at 100% B followed by a 20 min equilibration under standard conditions. The fraction containing deuterated tetrahydropapaverine (**13**) was collected and lyophilized. Deuterated tetrahydropapaverine (**13**) was obtained in 56% yield and completely free of papaverine (**1**) as confirmed by TLC, HPLC and high resolution LC–MS. The isotopic distribution of deuterium-labeled tetrahydropapaverine (**13**) was <0.1% [$^2H_0$]/ < 0.1% [$^2H_1$]/ < 0.1% [$^2H_2$]/ < 0.1% [$^2H_3$]/3% [$^2H_4$]/21.5% [$^2H_5$]/39% [$^2H_6$]/29.9% [$^2H_7$]/5.5% [$^2H_8$]/1% [$^2H_9$]. This observed isotopic distribution was largely in agreement with the expected isotopic distribution.

All other labeled or unlabeled alkaloids were from our laboratory collection.

The following equation was used for the calculation of the quotient between labeled and unlabeled alkaloid:

$$\frac{\text{nmol of labeled alkaloid}}{\text{nmol of labeled and unlabeled alkaloid}} = Q$$

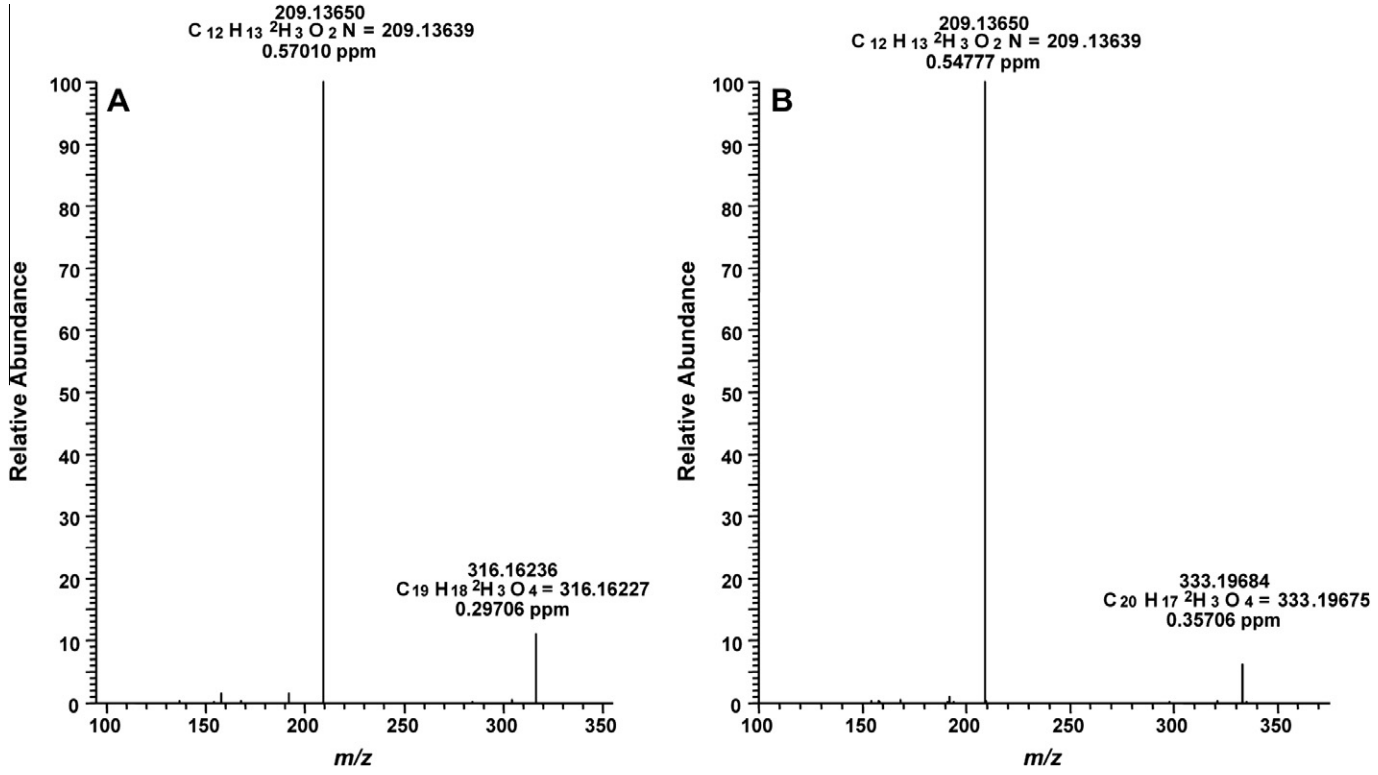

Fig. 6. High resolution MS/MS fragmentation spectra of synthesized [7-D_3]-laudanine (**11**) (A) and synthesized [3′,7-D_6]-laudanosine (**12**) (B).

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References

Battersby, A.R., 1963. Tilden lecture: the biosynthesis of alkaloids. Proc. Chem. Soc., 189–200.

Battersby, A.R., Binks, R., 1960. Biosynthesis of morphine: formation of morphine from norlaudanosoline. Proc. Chem. Soc., 360–361.

Battersby, A.R., Harper, B.J.T., 1959. Biogenesis of papaverine. Proc. Chem. Soc. 152.

Battersby, A.R., Harper, B.J.T., 1962. Alkaloid biosynthesis. Part I. The biosynthesis of papaverine. J. Chem. Soc., 3526–3533.

Battersby, A.R., Binks, R., Francis, R.J., McCaldin, D.J., Ramuz, H., 1964. Alkaloid biosynthesis. Part IV. 1-Benzylisoquinolines as precursors of thebaine, codeine, and morphine. J. Chem. Soc., 3600–3610.

Battersby, A.R., Foulkes, D.M., Brinks, R., 1965. Alkaloid biosynthesis. Part VIII. Use of optically active precursors for investigations on the biosynthesis of morphine alkaloids. J. Chem. Soc. (C), 3323–3332.

Battersby, A.R., Sheldrake, P.W., Staunton, J., Summers, M.C., 1977. Stereospecificity in the biosynthesis of papaverine. Bioorg. Chem. 6, 43–47.

Brochmann-Hanssen, E., Furuya, T., 1964. New opium alkaloid. J. Pharm. Sci. 53, 575.

Brochmann-Hanssen, E., Nielsen, B., Utzinger, G.E., 1965. Opium alkaloids. II. Isolation and characterization of codamine. J. Pharm. Sci. 54, 1531–1532.

Brochmann-Hanssen, E., Fu, C.C., Leung, A.Y., Zanati, G., 1971. Opium alkaloids. X. Biosynthesis of 1-benzylisoquinolines. J. Pharm. Sci. 60, 1672–1676.

Brochmann-Hanssen, E., Chen, C.-H., Chen, C.R., Chiang, H.-C., Leung, A.Y., McMurtrey, K., 1975. Opium alkaloids. Part XVI. The biosynthesis of 1-benzylisoquinolines in *Papaver somniferum*. Preferred and secondary pathways; stereochemical aspects. J. Chem. Soc. Perkin I, 1531–1537.

Brochmann-Hanssen, E., Chen, C.-Y., Linn, E.E., 1980. Biosynthesis of unnatural papaverine derivatives in *Papaver somniferum*. J. Nat. Prod. 43, 736–738.

Frenzel, T., Zenk, M.H., 1990a. (*S*)-Adenosyl-L-methionine:3′-hydroxy-*N*-methyl-(*S*)-coclaurine-4′-*O*-methyl-transferase, a regio- and stereoselective enzyme of the (*S*)-reticuline pathway. Phytochemistry 29, 3505–3511.

Frenzel, T., Zenk, M.H., 1990b. Purification and characterization of three isoforms of *S*-adenosyl-L-methionine: (*R*,*S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferase from *Berberis koetineana* cell cultures. Phytochemistry 29, 3491–3497.

Kutchan, T.M., 1998. Molecular genetics of plant alkaloid biosynthesis. In: Cordell, G.A. (Ed.), The Alkaloids, vol. 50. Academic Press, San Diego, pp. 257–316.

Lu, S.-T., Wu, Y.-C., Leou, S.-P., 1987. The oxidation of isoquinoline alkaloids with *m*-chloroperbenzoic acid. J. Chin. Chem. Soc. 34, 33–42.

Merck, G., 1848. Vorläufige Notiz über eine neue organische Base im Opium. Ann. Chem. 66, 125–128.

Ounaroon, A., Decker, G., Schmidt, J., Lottspeich, F., Kutchan, T.M., 2003. (*R*,*S*)-Reticuline 7-*O*-methyltransferase and (*R*,*S*)-norcoclaurine 6-*O*-methyltransferase of *Papaver somniferum* – cDNA cloning and characterization of methyl transfer enzymes of alkaloid biosynthesis in opium poppy. Plant J. 36, 808–819.

Phillipson, J.D., Handa, S.S., El-Dabbas, S.W., 1976. *N*-oxides of morphine, codeine and thebaine and their occurrence in *Papaver* species. Phytochemistry 15, 1297–1301.

Pienkny, S., Brandt, W., Schmidt, J., Kramell, R., Ziegler, J., 2009. Functional characterization of a novel benzylisoquinoline *O*-methyltransferase suggests its involvement in papaverine biosynthesis in opium poppy (*Papaver somniferum* L.). Plant J. 60, 56–67.

Robinson, R., 1917. A theory of the mechanism of the phytochemical synthesis of certain alkaloids. J. Chem. Soc. Trans. (Lond.) 111, 876–899.

Spenser, I.D., 1969. The biosynthesis of alkaloids and other nitrogenous secondary metabolites. In: Florkin, M., Stotz, E.H. (Eds.), Comprehensive Biochemistry, vol. 20. Elsevier Publishing Company, Amsterdam, pp. 231–413.

Stadler, R., Zenk, M.H., 1990. A revision of the generally accepted pathway for the biosynthesis of the benzyltetrahydroisoquinoline alkaloid reticuline. Liebigs Ann. Chem. 1990, 555–562.

Stadler, R., Kutchan, T.M., Loeffler, S., Nagakura, N., Cassels, B., Zenk, M.H., 1987. Revision of the early steps of reticuline biosynthesis. Tetrahedron Lett. 28, 1251–1254.

Taylor, E.P., 1952. Synthetic neuromuscular blocking agents. Part II. Bis(quaternary ammonium salts) derived from laudanosine. J. Chem. Soc., 142–145.

Taylor, E.C., Martin, S.F., 1974. A general method of alkylation and alkenylation heterocycles. J. Am. Chem. Soc. 96, 8095–8102.

Uprety, H., Bhakuni, D.S., Kapil, R.S., 1975. Biosynthesis of papaverine. Phytochemistry 14, 1535–1537.

Wat, C.K., Steffens, P., Zenk, M.H., 1986. Partial purification and characterization of (*S*)-adenosyl-L-methionine:norreticuline-*N*-methyltransferases from *Berberis* cell suspension cultures. Z. Naturforsch. 41c, 126–134.

Wickens, J.R., Sleeman, R., Keely, B.J., 2006. Atmospheric pressure ionisation mass spectrometric fragmentation pathways of noscapine and papaverine revealed by multistage mass spectrometry and in-source deuterium labelling. Rapid Commun. Mass Spectrom. 20, 473–480.

Winterstein, E., Trier, G., 1910. Die Alkaloide. Verlag Gebrüder Bornträger, Berlin.

Wu, W.-N., Huang, C.-H., 2006. Structural elucidation of isoquinoline, isoquinolone, benzylisoquinoline, aporphine, and phenanthrene alkaloids using API-ionspray tandem mass spectrometry. Chin. Pharm. J. 58, 41–55.