Cryptic Stereochemistry of Berberine Alkaloid Biosynthesis

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Abstract: The cryptic stereochemistry of one-carbon transformations in berberine biosynthesis has been elucidated by the chiral methyl group methodology and tritium NMR spectroscopy. The transfer of the methyl group of AdoMet to oxygen and nitrogen occurs with complete inversion of configuration. The oxidative formation of the berberine bridge from the *N*-methyl group of reticuline involves removal of a methyl hydrogen with a primary kinetic isotope effect $k_H/k_D = 4.0$ and its replacement by the phenyl group in an inversion mode. The subsequent aromatization catalyzed by (*S*)-tetrahydroprotoberberine oxidase (STOX) involves nonstereospecific hydrogen removal from C-8 with little or no isotope effect. In the formation of the methylenedioxy bridge, a hydrogen is removed from the methoxy group with $k_H/k_D > 5$ and replaced by the adjacent phenolic oxygen with apparent retention, accompanied by substantial racemization. The subsequent reductive opening of the methylenedioxy bridge of berberine to the methoxy group of jatrorrhizine proceeds stereospecifically, apparently in an inversion mode. Mechanistic interpretations of these findings are discussed.

Background

Berberine (**6**) is a quaternary isoquinoline alkaloid having antibacterial, antimalarial, and antipyretic activity. It is present in a variety of plant families, and considerable effort has gone into developing plant cell cultures that produce berberine.[1] The enzymology of berberine biosynthesis is now understood, and all of the biosynthetic enzymes have been isolated and characterized[2−8] (Figure 1). It is known that the immediate precursor of protoberberine alkaloids in plants is *(S)*-reticuline (**2**). The biosynthetic pathway from tyrosine to *(S)*-reticuline has been recently revised.[4,9] Broad substrate specificity for some of the biosynthetic enzymes forming reticuline had complicated the effort to elucidate the exact pathway. However, *(S)*-norcoclaurine (**1**) is now established as the true intermediate leading to reticuline.[10] Reticuline is converted into berberine in four steps. The four enzymes responsible have all been isolated and characterized.[8,11−14] Due to an erroneous assay system, it was first thought that columbamine is the substrate of the methylenedioxy bridge-forming enzyme.[15] But subsequently it was found that this so-called "berberine synthase" was a peroxidase,[16] and the true methylenedioxy bridge-forming enzyme is a cytochrome P-450 enzyme, named "canadine synthase", using (*S*)-tetrahydrocolumbamine (**4**) as the sole substrate[8] in both Berberidaceae and Ranunculaceae cell cultures.

With such a foundation laid, it is now possible to investigate more subtle and stereochemical questions concerning berberine biosynthesis. In the present work, the cryptic stereochemistry associated with the formation of the berberine bridge and the methylenedioxy bridge in this alkaloid has been determined. Preliminary accounts of parts of this work have previously appeared.[17,18]

Results and Discussion

Stereochemistry of Berberine Bridge Formation. The first step on the pathway from reticuline to berberine involves the formation of the berberine bridge. Thirty years ago, the groups of Barton[19] and Battersby[20] demonstrated that the berberine bridge (C-8 of scoulerine **3**) arises from an oxidative cyclization of the *N*-methyl group of reticuline (**2**). To investigate the stereochemistry of the berberine bridge formation, it was first

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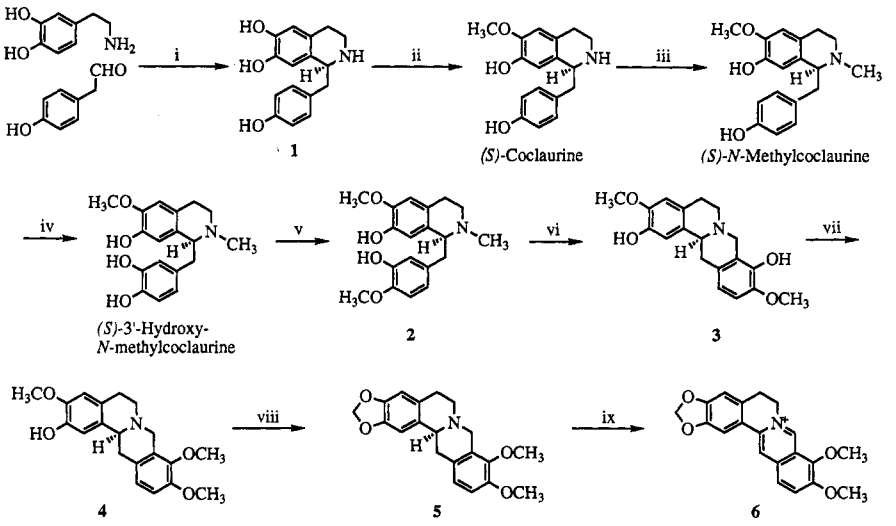
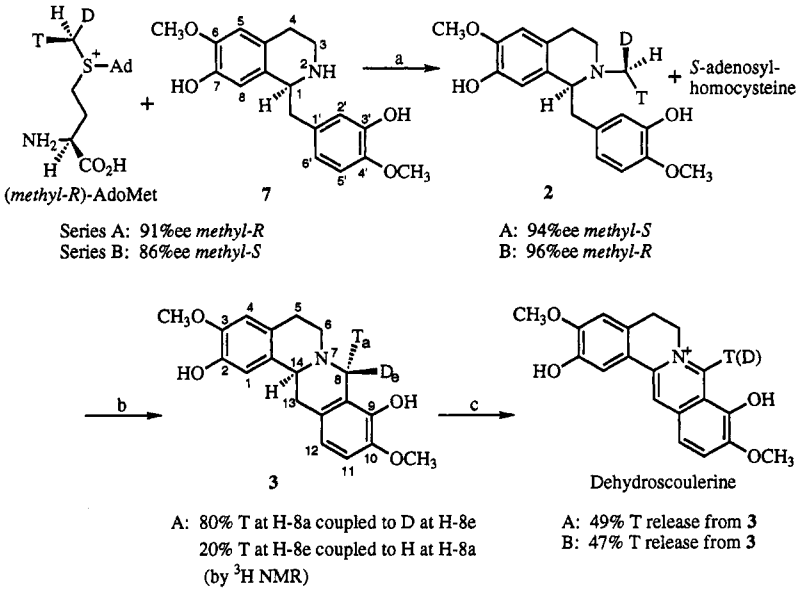


(i) *(S)*-Norcoclaurine synthase. (ii) *(S)*-Norcoclaurine-6-*O*-methyltransferase. (iii)*(R,S)*-Tetrahydrobenzylisoquinoline-*N*-methyltransferase. (iv) Phenolase. (v) 3'-Hydroxy-*N*-methyl-*(S)*-coclaurine-4'-*O*-methyltransferase. (vi) Berberine bridge enzyme. (vii) *(S)*-Scoulerine-9-*O*-methyltransferase. (viii) *(S)*-Canadine synthase. (ix) *(S)*-Tetrahydroprotoberberine oxidase (STOX).

Figure 1. Biosynthetic pathway to berberine in *Berberis* cell cultures.

Scheme 1[a]

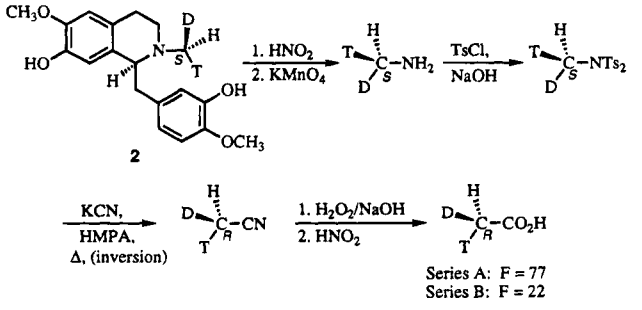


[a] (a) (*R,S*)-Tetrahydrobenzylisoquinoline-*N*-methyltransferase; (b) berberine bridge enzyme (BBE); (c) (*S*)-tetrahydroprotoberberine oxidase (STOX).

necessary to establish the stereochemistry of the *N*-methylation catalyzed by the enzyme *S*-adenosyl-L-methionine: (*R,S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferase.[21,22] Accordingly, *S*-adenosyl-*(methyl-R)*- and -*(methyl-S)*-[*methyl*-$^2H_1$,^3H]-L-methionine (AdoMet) were synthesized[23] from chiral acetate and used as enzyme substrates for the methyltransferase reaction (Scheme 1).

Norreticuline (**7**) and *(methyl-R)*-[*methyl*-$^2H_1$,^3H]AdoMet (100 mCi/mmol, 91% ee) were incubated with the enzyme *S*-adenosyl-L-methionine: (*R,S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferase to produce *(S)*-reticuline (**2**). An aliquot of the resulting reticuline was then degraded as shown (Scheme 2). Oxidation of the reticuline with $KMnO_4$ generated methylamine from the *N*-methyl group. Methylamine was next tosylated twice to give methylamine *N,N*-ditosylate. This compound then underwent nucleophilic displacement by KCN with an inversion of configuration of the methyl group. The resulting acetonitrile was then converted under mild conditions to acetamide, which was diazotized to give acetic acid. Chirality

Scheme 2



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analysis of the acetic acid by the method of Cornforth[24] and Arigoni[25] gave an *F* value[26,27] of 77, indicating that the methyl group in the acetic acid had a 94% ee of the *R* configuration. Since the degradation involved one inversion step the methyl group was of the *S* configuration in reticuline. Therefore, the methyl group of AdoMet was transferred to the nitrogen of norreticuline with clean inversion of configuration. This is consistent with the observations on most other methyltransferases.[27-29] The complementary set of methylation experiments were performed with *(methyl-S)*-[*methyl*-$^2H_1$,^3H]-AdoMet (160 mCi/mmol, 86% ee). Degradation of an aliquot of the resulting *(S)*-reticuline gave acetic acid that had an *F* value of 22 (96% ee of *S*). Again, since there was one inversion step in the degradation, this indicated that the methyl group of AdoMet was transferred to norreticuline with inversion of configuration.

The reticulines generated, as described above, possess a chiral methyl group on the nitrogen (one sample of each configuration). These were then used in conjunction with the biosynthetic enzymes to determine the cryptic stereochemistry of berberine bridge formation. Berberine bridge enzyme[11,12] (BBE) catalyzes the conversion of *(S)*-reticuline (**2**) into scoulerine (**3**). Both samples of chirally labeled reticuline were subjected to the sequential action of BBE and then *(S)*-tetrahydroprotoberberine oxidase[14,30] (STOX), and the release of tritium into the medium was monitored. STOX oxidizes scoulerine, removing one hydrogen from the newly formed methylene group (C-8) of scoulerine. It was found that BBE releases from each of the chirally labeled substrates only 8% of the tritium (Figure 2). This indicates that there is a significant primary kinetic isotope effect in the abstraction of a hydrogen from the *N*-methyl group. Upon addition of STOX, nearly half of the remaining tritium was released into the medium for both substrates. From these results, it follows that either BBE or STOX or both enzymes act nonstereospecifically at the labeled center. Additionally, hydrogen abstraction from C-8 of scoulerine by STOX must proceed with little or no isotope effect.

To gain further insight into the cryptic stereochemistry of BBE, tritium NMR spectroscopy was used. The goal was to establish the configuration of the tritiated methylene group (C-8 of **3**) produced by the action of BBE. To this end, a larger sample of labeled scoulerine hydrochloride (153 μCi after purification) was prepared. The scoulerine (**3**) was again formed enzymatically from *(methyl-R)*-[*methyl*-$^2H_1$,^3H]AdoMet (800 μCi, 86% ee) and norreticuline (**7**), methylating first with coclaurine-*N*-methyltransferase, followed by ring closure using BBE (Scheme 1). The ^1H,^2H-decoupled tritium NMR spectrum (Figure 3) of the resulting scoulerine showed two signals, one at δ 4.76 ppm (20% of integration) and the other at δ 4.29 ppm (80% of integration). Using an unlabeled sample of enzymatically prepared scoulerine, we unequivocally assigned the signals of the methylene protons at C-8. The two methylene protons occupy an axial (H-8a, δ_{1H} 4.34 ppm) or equatorial (H-8e, δ_{1H} 4.76 ppm) position with respect to the ring system. A 2D phase sensitive NOESY[31] experiment and a 1D steady-state NOE

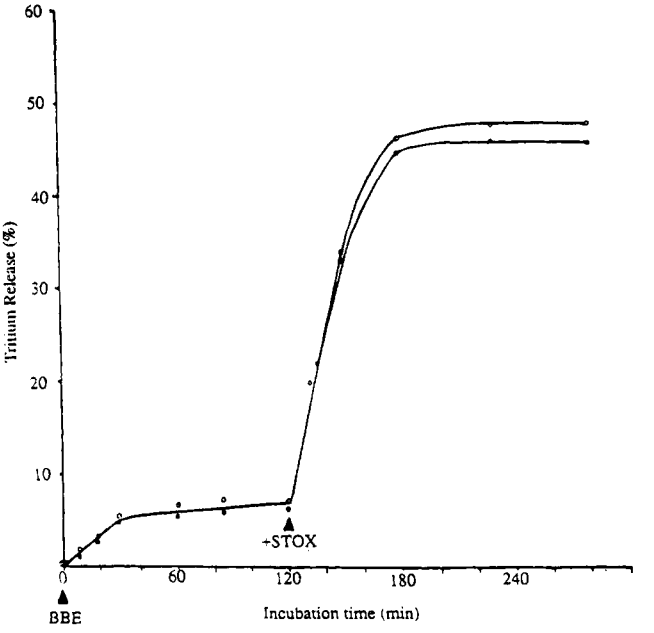

Figure 2. Tritium release in the formation of the berberine bridge by BBE and its dehydrogenation by STOX with *(methyl-R)*-[*methyl*-$^2H_1$,^3H]-**2** (●) and *(methyl-S)*-[*methyl*-$^2H_1$,^3H]-**2** (○) as substrate. The incubations contained 4.2 nmol of **2** (0.67 and 0.42 μCi, respectively) and 0.25 pkat of BBE. Samples were analyzed for tritium released into the solvent at the times indicated. At 120 min, 5 pkat of STOX were added, and the analyses were continued.

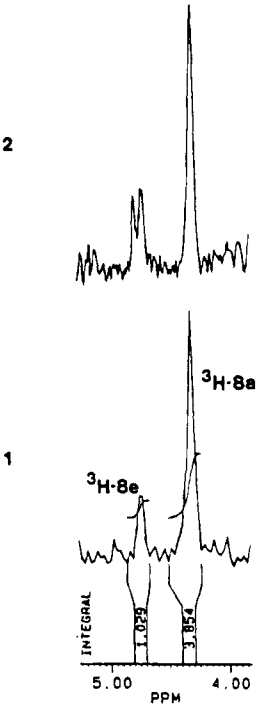

Figure 3. Tritium NMR spectra of scoulerine (**3**) generated from *(methyl-S)*-[*methyl*-$^2H_1$,^3H]-**2** with BBE. Sample contained 153 μCi ^3H, solvent CD_3OD, repetition time 1.0 s; spectrum 1, composite pulse broadband ^1H-decoupled, 76472 acquisitions; spectrum 2, ^1H-decoupler gated off during acquisition, WALTZ-16 ^2H broadband decoupled, 47 347 acquisitions.

difference spectrum clearly showed that only the signal for the axial proton (H-8a) undergoes cross-relaxation with H-14a and

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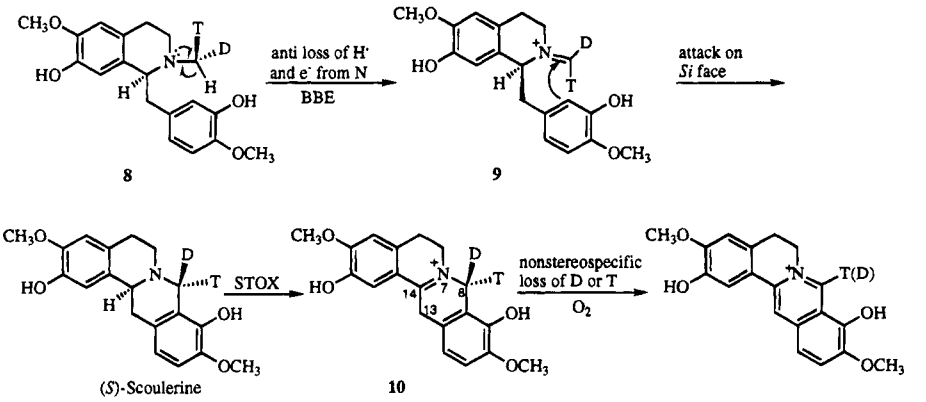
Scheme 3

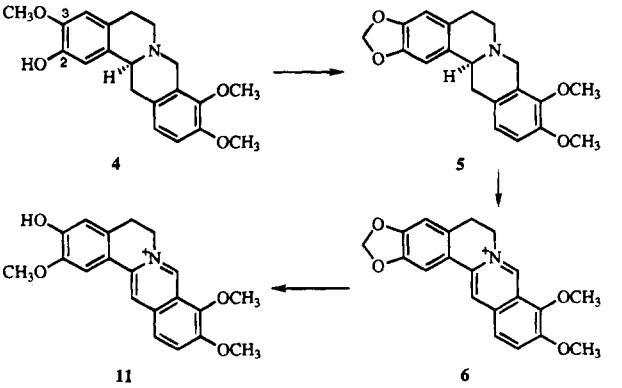


H-6a. These assignments were in accord with anisotropy arguments presented in the earlier literature,[32,33] and the coupling constants were consistent with the B/C *trans*-tetrahydroprotoberberine configuration. From the integration of the tritium NMR spectrum, it was clear that 80% of the tritium was in the axial *pro-R* position and 20% occupied the equatorial *pro-S* position in the labeled scoulerine. Upon removal of the broadband proton decoupling, the ^3H NMR signal at δ 4.76 ppm split into a doublet (J = 15.3 Hz) whereas the signal at δ 4.29 ppm remained unchanged. This indicated that when tritium was present in the axial position, there was a geminal deuterium in the equatorial position, which caused a 0.05 ppm upfield isotope shift of the ^3H resonance. However, molecules containing an equatorial tritium had an axial, geminal hydrogen that coupled with the tritium and caused observable splitting.

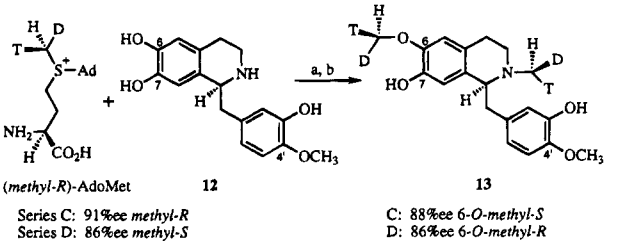
It follows from the data that BBE operates highly or completely stereospecifically, replacing an *N*-methyl hydrogen by the phenyl group in an inversion mode, and that the hydrogen abstraction involves a primary kinetic isotope effect ($k_H/k_D \approx 4$). One possible mechanism for BBE consistent with the data (Scheme 3) would involve the removal of an electron from the nitrogen with subsequent loss of a hydrogen atom from the *N*-methyl group in an *anti* fashion (relative to the original nitrogen lone pair). Starting from (*methyl-S*)-[*N*-*methyl*-^{2}H,^{3}H]-reticuline (**8**), the above process would generate the (*Z*)-methyleniminium ion (**9**), which could then condense with C-2 of the phenyl ring. If there is no rotation about the iminium double bond, then the stereochemistry at C-14 of reticuline would dictate that the condensation must occur stereospecifically on the *si* rather than on the *re* face of the methylene carbon of the methyleniminium ion.

The observation of nonstereospecific hydrogen removal from C-8 in the aromatization of ring C is consistent with the previously proposed mechanism for this aromatization process.[14] On the basis of the observed stoichiometry, 2 mol of O_2 consumed and 1 mol of H_2O_2 produced/mol of substrate, it has been suggested that the enzyme STOX only catalyzes the dehydrogenation of the substrate to the 7,14 iminium ion (**10**). This species can then undergo spontaneous air oxidation to generate the aromatic C ring. Related work by Battersby and co-workers[34] also supports this proposal. They found that scoulerine, stereospecifically tritiated at C-13, was converted in *Chelidonium majus* plants into berberine and coptisine with "extensive and nonstereospecific loss of hydrogen from C-13" (82−86% of the tritium was lost instead of the expected 50%).

Scheme 4



Scheme 5[a]



[a] (a) (*S*)-Norcoclaurine-6-*O*-methyltransferase; (b) (*R,S*)-tetrahydrobenzylisoquinoline-*N*-methyltransferase.

Reversible interconversion between the 7,14 iminium ion (**10**) and the 13,14-enamine can account for the greater than expected loss or washout of tritium from C-13. If the tautomerization between **10** and the enamine is reversible and nonenzymatic, it may proceed through many cycles before the C ring is finally aromatized. This would cause significant, nonstereospecific loss of tritium from C-13 as was observed by Battersby's group.

Stereochemistry of *O*-Methyl Group Formation in Jatrorrhizine Biosynthesis. The successful use of tritium NMR, in determining the stereochemistry of berberine bridge formation, led us to attempt the same approach to investigate the stereochemistry of the methylenedioxy bridge formation in berberine biosynthesis. It was known from previous labeling experiments[35] that the methylenedioxy bridge arises from an oxidative cyclization involving the phenolic *O*-methyl group and the adjacent hydroxy group of tetahydrocolumbamine (**4**) in *Berberis* cell cultures (Scheme 4). In a further transformation, these cell cultures can form berberine and then open the methylenedioxy bridge of berberine (**6**) to produce the alkaloid jatrorrhizine[35,36] (**11**). This is the major alkaloid produced in *Berberis*

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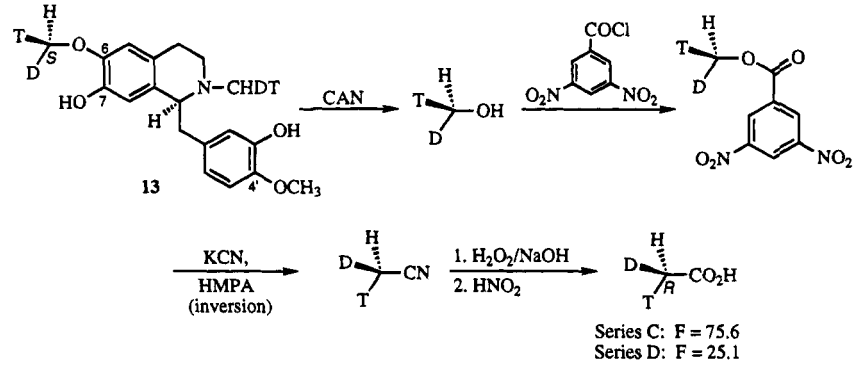
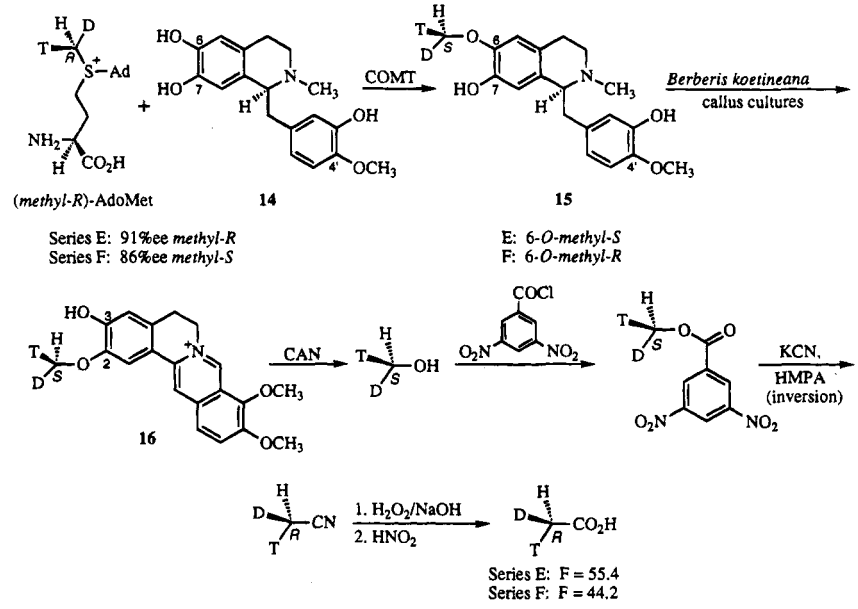
Scheme 6



Scheme 7



cell cultures. The ring opening presumably occurs by hydride displacement of a phenolic oxygen. The positively charged nitrogen of berberine could assist this displacement by acting as an electron sink. The net result of this sequence of reactions is a methyl group migration from O-3 of tetrahydrocolumbamine (**4**) to the O-2 position of jatrorrhizine (**11**).

Before the methylenedioxy bridge was observed by tritium NMR, the stereochemical fate of a chiral methyl group was followed from AdoMet through the above sequence of biosynthetic transformations. Samples of (1*S*)-[6-*O*,*N*-*methyl*-^2H,^3H]-reticuline (**13**) were prepared from (*methyl-R*)- and (*methyl-S*)-[*methyl*-$^2H_1$,^3H]AdoMet and 4′-*O*-methylnorlaudanosoline (**12**) using the biosynthetic 6-*O*-[37] and -*N*-methyltransferases[5] isolated from *Berberis* cell cultures (Scheme 5). The resulting labeled reticulines (**13**) were degraded to convert the 6-*O*-methyl group stereospecifically into the methyl group of acetic acid for chirality analysis (Scheme 6). The labeled reticuline produced from (*methyl-R*)-[*methyl*-$^2H_1$,^3H]AdoMet (100 mCi/mmol, 91% ee) generated acetic acid with an *F* value of 75.6 upon degradation. Since the degradation sequence involved one inversion of configuration, this value indicates an 88% ee of the *S* configuration for the 6-*O*-methyl group of the reticuline. Therefore, the methyl group was transfered from AdoMet by the enzyme (*S*)-norcoclaurine-6-*O*-methyltransferase with inversion of configuration. Degradation of labeled reticuline produced from (*methyl-S*)-[*methyl*-$^2H_1$,^3H]AdoMet (160 mCi/mmol, 86% ee) converted the 6-*O*-methyl group into acetic acid that had an *F* value of 25.1, indicating an 86% ee of the *R* configuration for the reticuline *O*-methyl group. This complementary experiment again showed methyl transfer to occur with inversion of configuration.

The *in vivo* formation of the 2-*O*-methyl group of jatrorrhizine (**11**) from the 6-*O*-methyl group of reticuline was studied in callus cultures of *Berberis koetineana* (Scheme 7). (1*S*,*methyl-R*)- And (1*S*,*methyl-S*)-[6-*O*-*methyl*-^2H,^3H]reticulines (**15**) were synthesized using the above samples of chiral AdoMet, (1*S*)-4′-*O*-methyllaudanosoline (**14**), and porcine liver catechol *O*-methyltransferase (COMT). It has been shown that COMT transfers the methyl group of AdoMet with inversion of configuration.[38] The two labeled reticuline samples were purified to remove any traces of the 7-*O*-methyl isomer and fed to the callus cultures. The resulting samples of labeled jatrorrhizine (**16**) were purified to remove traces of any other alkaloids, particularly columbamine, and then degraded. The acetic acid obtained from the two samples gave *F* values of 55.4 and 44.2, respectively. This corresponds to a 19% ee of the *S* configuration and a 20% ee of the *R* configuration for the 2-*O*-methyl group in the jatrorrhizine samples. Therefore, the biosynthetic transformation of tetrahydrocolumbamine (**4**) to canadine (**5**), berberine (**6**), and jatrorrhizine (**11**) has produced

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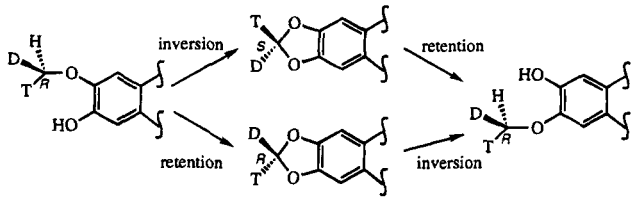
Scheme 8

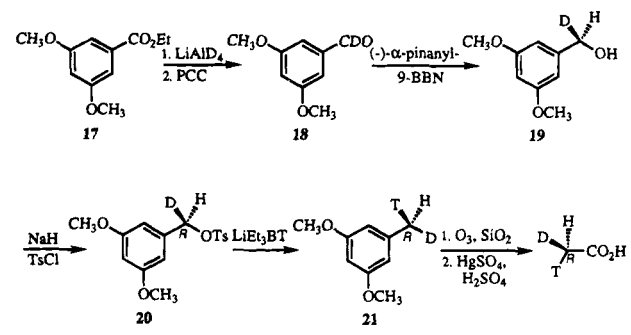


a methyl group in jatrorrhizine having predominantly the same configuration as was present in the methyl group of tetrahydrocolumbamine. To account for the observed stereochemistry of the overall transformation (**4** → **11**), there are two possible reaction pathways (Scheme 8). The first would require a ring-closing reaction that operated in a retention mode, followed by a ring-opening reaction in an inversion mode to complete the methyl migration.[39] The methyl migration is itself the equivalent of an additional inversion step. The second possible reaction pathway would involve a ring closure in an inversion mode and then a ring opening in a retention mode. In either case, one or the other reaction, or both, must involve substantial racemization.

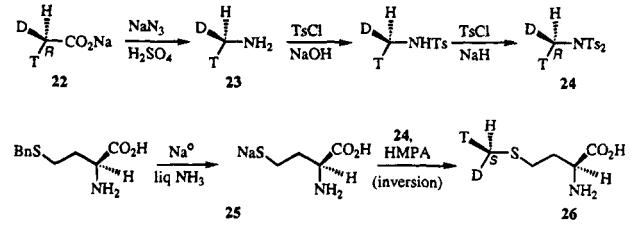
Stereochemistry of Methylenedioxy Bridge Formation. To distinguish between these two stereochemical reaction paths, tritium NMR was used to establish the configuration of the methylenedioxy bridge in berberine. However, certain problems had to be addressed to enable us to use this methodology. First, enough tritium had to be present in the methylenedioxy bridge of an NMR-sized sample to see the ^3H signals. We have determined that ~100−150 μCi of tritium per site in a molecule will allow detection in a reasonable amount of time (number of scans). High-berberine-producing cell cultures caused a natural dilution problem. The labeled berberine would be mixed with a large amount of indigenous unlabeled berberine, thereby reducing the overall tritium content in the amount of sample that, based on solubility, can be introduced into the NMR spectrometer. The synthesis and use of high specific activity chiral AdoMet could potentially overcome this problem. The second and more immediate concern was that the methylenedioxy bridge protons are magnetically equivalent in berberine. Creation of a chiral center at C-14 by reduction of the berberine made the methylene bridge protons diastereotopic. Unfortunately, due to their distance from the chiral center, they still had identical chemical shifts. Therefore, a derivative of the labeled berberine had to be found that would break this degeneracy and allow the observation of the exact location of tritium in the molecule. A series of derivatives that relied on alkylation of the nitrogen in the reduced berberine molecule to effect a chirality transfer that would reach to the methylenedioxy bridge protons were tried.

High specific activity sodium (*R*)-[2-$^2H_1$,^3H]acetate was required for the synthesis of (*methyl-S*)-[*methyl*-$^2H_1$,^3H]methionine (**26**). The chiral acetate was synthesized using a modification of the procedure described by Kobayashi *et al.*[40] (Scheme 9). Ethyl 3,5-dimethoxybenzoate (**17**) was reduced with $LiAlD_4$ to the deuterated alcohol and then oxidized to the aldehyde (**18**). Stereospecific reduction of **18** gave the chiral benzyl alcohol (**19**), which was next converted to the tosylate (**20**). Displacement of the tosyl group with carrier-free supertritiide[41] produced

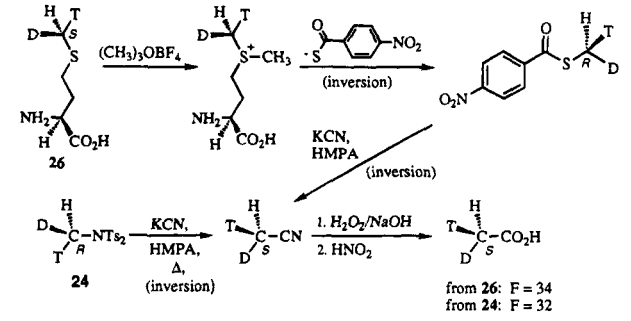
Scheme 9



Scheme 10



Scheme 11



the carrier-free (7*R*)-3,5-dimethoxy-[7-$^2H_1$,$^3H_1$]toluene (**21**). Addition of unlabeled **21** allowed the desired specific activity to be attained before ozonolysis to generate the chiral acetic acid (specific activity = 755 mCi/mmol).

High specific activity methionine (**26**) was then synthesized by the established route (Scheme 10),[23] first converting chiral sodium acetate (**22**) into the methylamine ditosylate (**24**) then displacing the ditosylamine with the sulfur anion of L-homocysteine (**25**). This S_N2 displacement causes inversion of the methyl group of **24**. The optical purity of the chiral methyl group in **26** depends upon the stereospecificity of the Schmidt reaction[42,43] in converting sodium acetate (**22**) into methylamine (**23**). Unfortunately, we have observed that this transformation does not always proceed with complete retention of configuration of the methyl group. A varying amount of racemization accompanies the reaction. As a result, the high specific activity methylamine ditosylate (**24**) that was prepared in this synthesis had a lower optical purity (62% ee of *methyl-R*) than the previously used samples. Also, the ^1H-coupled ^3H NMR of **24** indicated that it contained 15% CH_2T methyl groups. No racemization of the chiral methyl group occurs during the remaining steps of the synthesis.[44,45] Degradation of both **24** and **26** to acetic acid[45] followed by chirality analysis gave essentially the same *F* values (Scheme 11).

(39) The terms retention mode and inversion mode are defined as follows. A reaction operating in a retention mode replaces one ligand group for another at the same ligand site on the carbon atom with no change in the spacial orientation of the remaining ligand groups. Inversion mode refers to ligand substitution with a concomitant change in the spacial orientation (inversion) of the remaining ligands on the carbon atom.

(40) Kobayashi, K.; Jadhav, P. K.; Zydowsky, T. M.; Floss, H. G. *J. Org. Chem.* **1983**, *48*, 3510−3513.

(41) Andres, H.; Morimoto, H.; Williams, P. G. *J. Chem. Soc., Chem Commun.* **1990**, 627−628.

(42) Shioiri, T. In *Comprehensive Organic Synthesis*; Trost, B. M., Fleming, I., Eds.; Pergamon Press: Oxford, 1991; Vol. 6, pp 795−828 and references therein.

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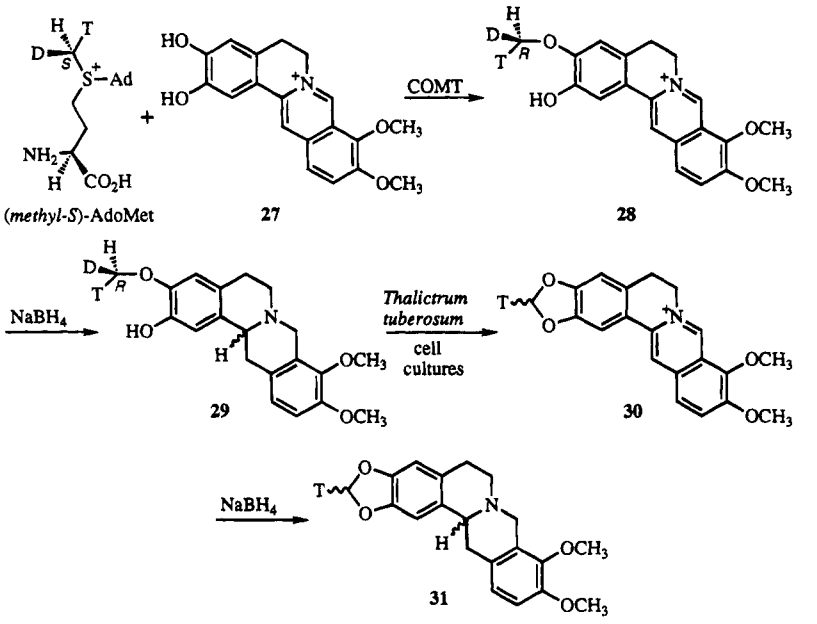
Scheme 12

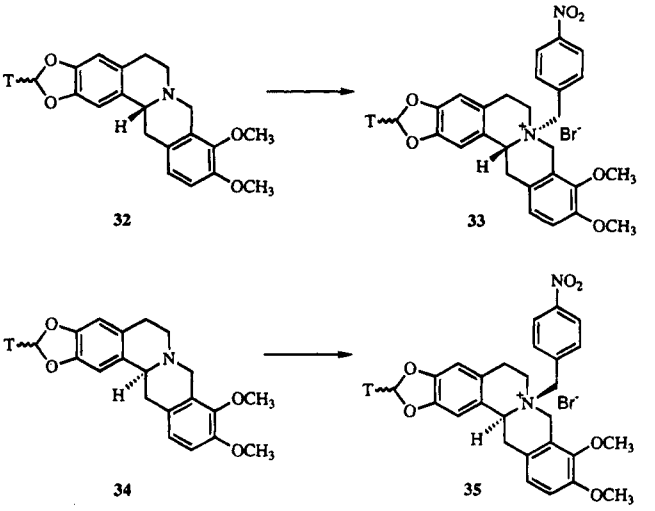


While the synthesis of (*methyl-S*)-[*methyl*-^{2}H,^{3}H]methionine (**26**) was underway, a stable cell line of *Thalictrum tuberosum* was developed in the Zenk laboratory. The cell line lacks two of the early biosynthetic enzymes on the pathway to berberine. Except for the two missing enzymes, all of the other biosynthetic machinery is intact in these cultures. The biosynthetic pathway to berberine in *T. tuberosum* is analogous to the pathway in *Berberis*. The advantage of using this cell line instead of *Berberis* cell cultures for the feeding experiment was the absence of indigenous berberine, which could cause tritium dilution problems.

The labeled compound fed to *T. tuberosum* cell cultures was (14*RS*,*methyl-R*)-[3-*O*-*methyl*-^{2}H$_1$,^{3}H]tetrahydrocolumbamine (**29**, Scheme 12). Labeled methionine (**26**) was enzymatically converted to (*methyl-S*)-[*methyl*-^{2}H$_1$,^{3}H]AdoMet[46] and used with COMT to methylate desmethyleneberberine (**27**). As before, this transfers the methyl group of AdoMet with inversion of configuration. The resulting (*methyl-R*)-[3-*O*-*methyl*-^{2}H$_1$,^{3}H]-columbamine (**28**) was converted into **29** by $NaBH_4$ reduction and fed to *T. tuberosum* cell cultures. The berberine (**30**) formed was then isolated and purified. Reduction of the isolated berberine with $NaBH_4$ gave (±)-canadine[47] (**31**) containing tritium in the methylenedioxy bridge.

Tritium NMR analysis of the (±)-canadine (**31**) showed that the enzyme (*S*)-canadine synthase operated with an observable primary kinetic deuterium isotope effect. The ^{1}H-decoupled ^{3}H NMR spectrum showed two signals, one at δ 5.97 ppm (15–20% of the total integrated area) and the other at δ 5.94 ppm (80–85% of the total integrated area). The signal at δ 5.97 ppm was due to tritium on the methylenedioxy bridge which had a geminal hydrogen (deuterium lost from the chiral methyl group), and the signal at δ 5.94 ppm resulted from tritium having a geminal deuterium ($\Delta\delta$ = 0.03 ppm) (hydrogen lost from the chiral methyl group). Correcting for the small amount of CH_2T that was present along with the chiral methyl group allowed for an estimation of the isotope effect to be $k_H/k_D > 5$. The two diastereotopic methylene hydrogens were, however, degenerate, presumably because they experience little effect of the distant chiral center at C-14.

Scheme 13



In order to use ^{3}H NMR to quantify the amount and position of tritium in the methylenedioxy bridge, it was necessary to resolve the labeled (±)-canadine (**31**) and to derivatize each enantiomer to break the degeneracy of the methylene bridge protons. Resolution was accomplished using HPLC on a chiral column. (14*R*)-(+)-Canadine (**32**) had a retention time of 14 min, while the enantiomer (**34**) eluted after 44 min. The absolute configuration of the two enantiomers rests firmly on earlier work in the literature.[48] The most effective derivative of canadine was determined by synthesizing a series of quaternary ammonium salts with alkyl groups of varying size and functionality. The *p*-nitrobenzyl derivative proved best at relaying the effects of the chirality present at C-14 so that they were felt at the methylenedioxy bridge. Each enantiomer of labeled canadine was derivatized with *p*-nitrobenzyl bromide to form the quaternary ammonium salts (**33**, **35**) having a *trans* relationship between the hydrogen at C-14 and the *p*-nitrobenzyl group on the adjacent nitrogen (Scheme 13). The ^{1}H NMR spectra of these derivatives (**33**, **35**) show a separate resonance

(45) Zydowsky, T. M.; Courtney, L. F.; Frasca, V.; Kobayashi, K.; Shimizu, H.; Yuen, L.-D.; Matthews, R. G.; Benkovic, S. J.; Floss, H. G. *J. Am. Chem. Soc.* **1986**, *108*, 3152–3153.

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(47) Canadine is also referred to as tetrahydroberberine in the literature.

(48) Corrodi, H.; Hardegger, E. *Helv. Chim. Acta* **1956**, *39*, 889–897.

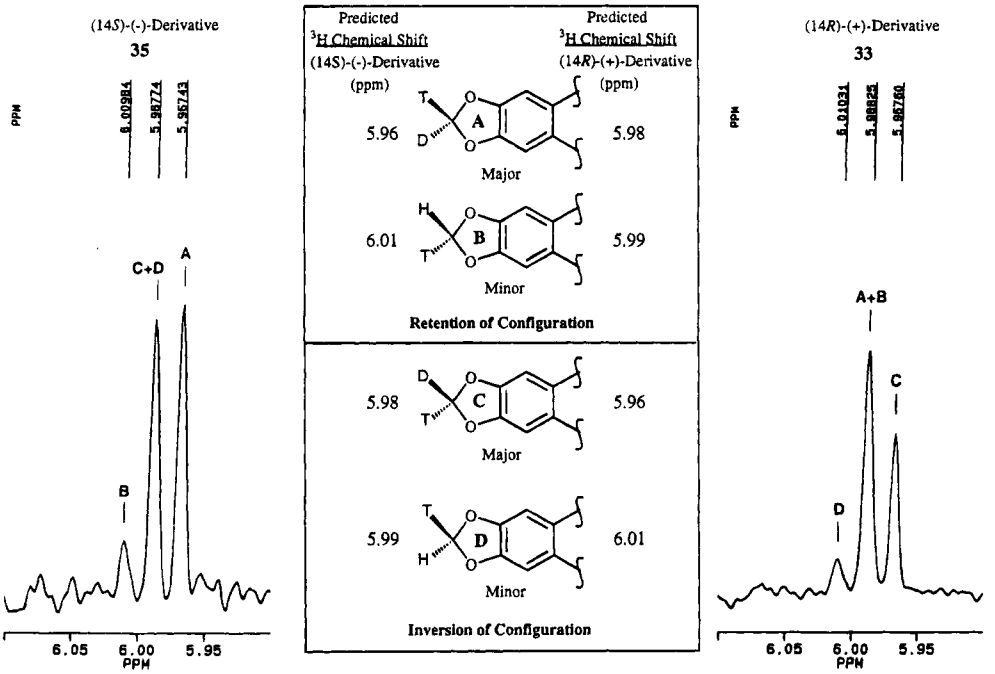


Figure 4. Proton-decoupled tritium NMR spectra of (+)- and (−)-canadine derivatives **33** and **35**. The samples contained 270 and 300 μCi of tritium, respectively; the solvent was $CDCl_3$. Predicted ^3H chemical shifts are based on the ^1H chemical shifts and the observed deuterium isotope shift is based on the ^3H signal in **31**.

for each methylenedioxy bridge proton. The *p*-nitrobenzyl group is expected to shift the resonance of the bridge proton on the same side of the ring system upfield (0.02 ppm) from the other bridge proton, and consistent with this prediction, we once observed an NOE between the upfield methylene proton and the nitrophenyl ring protons. However, the distance between these protons is at the limits of what can be detected by NOE measurements, and several attempts to reproduce this NOE gave negative results. Thus, the configurational assignment of the two methylenedioxy protons is only tentative.

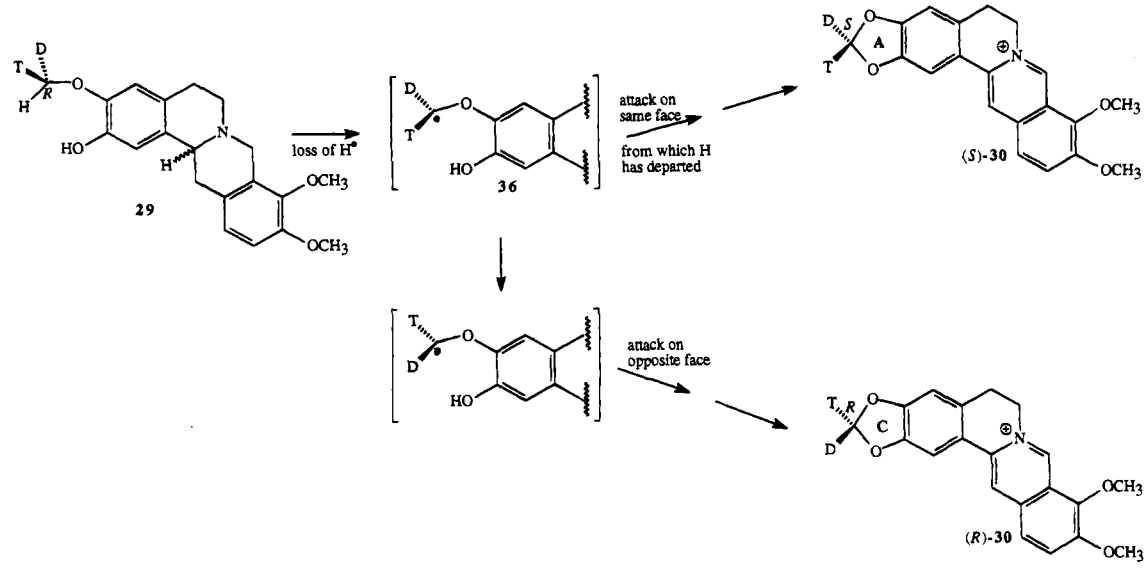
The ^1H-decoupled ^3H NMR spectrum of the 14*R* derivative (**33**) shows three distinct signals at δ 6.01, 5.98, and 5.96 with a 0.28:1.66:1.00 area ratio (Figure 4). The 14*S* derivative (**35**) shows three signals at the same chemical shifts but with a 0.29: 0.98:1.00 area ratio. Principally, there could be four different tritiated species (**A**−**D**). The tritium could occupy either the *pro-R* or *pro-S* position on the methylene bridge and have either a geminal deuterium or hydrogen. The ^3H signal will have a different chemical shift for each of these possible combinations. The observed deuterium isotope shift of a ^3H signal when a geminal deuterium is present is 0.03 ppm upfield from the signal with a geminal hydrogen. For each enantiomeric derivative, the central two of the four possible signals are not resolved ($\Delta\delta = 0.01$ ppm) and the observed spectrum shows three peaks.

If complete racemization occurred during the formation of the methylenedioxy bridge, then the integrated area for the ^3H signals of **A** + **B** would equal the integrated area of **C** + **D**. For both enantiomers, the ^1H-decoupled ^3H NMR spectra indicate that species **A** and **B** are present in greater amounts than **C** and **D**. Assuming the tentative assignment for the two methylendioxy protons to be correct, it is clear that **A** and **B** both arise from a direct (retention mode) substitution of the phenolic oxygen at C-2 of tetrahydrocolumbamine (**4**) for the hydrogen (or deuterium) that was removed from the chiral methyl group. For example, a hydrogen must be removed from the chiral methyl group to generate **A**. The newly formed C−O bond must then occupy the same ligand site on the carbon that previously held the C−H bond. Therefore, the oxidative ring-closing reaction has occurred predominantly in a retention mode. Species **C** and **D** result from a racemization process that occurs during the formation of the methylenedioxy bridge.

An overall methyl group composition of 12% CH_2T, 14% *methyl-S*, and 74% *methyl-R* for the labeled tetrahydrocolumbamine (**29**) was deduced from the degradation and configurational analysis of the methyl group (Scheme 11) and the ^3H NMR analysis of the starting methionine. To simplify the analysis of the methylenedioxy bridge-forming reaction, let us consider only the case of a chiral methyl group losing a hydrogen. Under these conditions, only **A** and **C** will be formed. The observed ratio **A/C** was 1.3 as calculated from the ^3H NMR spectra of both derivatives. This observed ratio is slightly lower than the theoretical one expected from optically pure **29** because of the effect of the presence of some *methyl-S* enantiomer in the labeled tetrahydrocolumbamine that was used as the substrate. The theoretical ratio **A/C** was calculated to be 1.5 when adjusted to account for the (*methyl-S*)-tetrahydrocolumbamine.[49] This ratio reflects a 20% ee of the configuration resulting from a retention mode cyclization during the formation of the methylenedioxy bridge. Therefore, the methylenedioxy bridge has been formed by an oxidative cyclization that operates predominantly in a retention mode. This is in excellent qualitative and quantitative agreement with the results observed for jatrorrhizine biosynthesis described above and indicates that the two-step process involving the formation of the methylenedioxy bridge followed by its opening occurs in a retention mode for the cyclization and an inversion mode for the ring opening. The data imply that all of the observed racemization occurs during the formation of the methylenedioxy bridge and by a process other than hydrogen exchange and that the second enzyme reaction that opens this bridge to form jatrorrhizine must operate stereospecifically and cause an inversion of configuration at the methylene bridge carbon. This is consistent with the suggested mechanism for the ring-opening reaction.[18,36]

(49) This calculation makes the plausible assumption that (*S*)-canadine synthase operates on both *R* and *S* methyl groups in an identical fashion.

Scheme 14



Mechanistically, the enzymatic formation of the methylenedioxy bridge could be analogous to the formation of the berberine bridge. Loss of an electron and a hydrogen atom would form an oxonium ion intermediate that could ring close with the adjacent hydroxyl group. The observed partial racemization can then be explained by the lower barrier to rotation of the methylene oxonium ion compared to the methylene iminium ion that has been proposed for berberine bridge formation. Any rotation about the C−O bond of the oxonium ion will result in racemization. Alternatively, or in addition, there could be less discrimination regarding the face of the oxonium ion involved in the condensation step. In the formation of the berberine bridge, the chiral center adjacent to the iminium ion dictates on which face of the iminium ion that condensation must occur.

The formation of the methylenedioxy bridge also shows similarities with other biosynthetic oxidative cyclization reactions. Isopenicillin N synthase[50] (IPNS), deacetoxycephalosporin C synthase[51,52] and clavaminate synthase[53−56] (CS) all catalyze oxidative cyclizations where an unactivated carbon−hydrogen bond is broken and a new carbon−heteroatom bond is formed. Both IPNS and CS operate by stereospecifically replacing the hydrogen with a heteroatom at the same ligand site on the carbon atom (retention mode). When the heteroatom involved is oxygen, mechanistic similarities to hydroxylation enzymes have been invoked.[55,57] Methylenedioxy bridge formation in berberine biosynthesis also replaces a C−H bond with a new C−O bond at the same ligand site on carbon. The fact that canadine synthase is a P-450 enzyme underscores this analogy. It is therefore perhaps more likely that the mechanism involves radical instead of ionic intermediates (Scheme 14) in keeping with the proposed mechanisms of the above enzymes. The substantial racemization observed in the ring-closure reaction would then reflect configurational inversion of the intermediate oxymethylene radical by rotation around the C−O bond prior to its reaction with the adjacent oxygen in the rebound reaction. A similar partial racemization, presumably *via* an intermediate alkyl radical, has recently been observed in the methane monooxygenase reaction.[58] The, at least tentative, assignment of predominant retention stereochemistry to the methylendioxy bridge formation is also in keeping with this interpretation.

Experimental Section

General Procedures. All NMR spectra were obtained on an IBM AF-300 spectrometer operating at a field strength of 7.1 T. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75.4 MHz, respectively. Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane with the solvent resonance as an internal reference. Coupling constants (J) are given in hertz (Hz). ^{3}H NMR spectra were recorded at 320.13 MHz using the above spectrometer equipped with a tritium channel and a dual ^{1}H/^{3}H 5 mm probe. Tritium spectra were referenced to the proton resonance of tetramethylsilane with a conversion factor of 1.066 639 74 for obtaining the equivalent tritium frequency. Analytical thin layer chromatography (TLC) was performed using Merck silica gel 60 F-254 plates (0.25 mm precoated on glass). Preparative TLC was performed using Merck silica gel 60 F-254 plates (1 and 2 mm precoated) or Macherey Nagel Polygram Sil G/UV$_{254}$ (0.25 mm precoated). Compounds were visualized under UV light or by spraying with a 2% solution of ninhydrin in ethanol, followed by heating. Flash chromatography[59] was performed using 230−400 mesh silica gel. The following HPLC systems were used. A Beckman single pump System Gold HPLC equipped with a Waters UV detector monitoring at 254 nm was used with a Chiralcel OD column (purchased from J. T. Baker Inc.) for enantiomer resolutions. Alkaloids from cell cultures or enzymatic incubations were purified using either an Abimed-Gilson 2 pump model 303 HPLC equipped with a mixer and a UV detector or a Waters-Millipore 2 pump model 510 HPLC equipped with an automated gradient programmer, a UV detector, and an integrator (data module 740). Radioactivity was measured in Atomlight (DuPont) liquid scintillation fluid using a Beckman LS-1801 liquid scintillation counter with [^{3}H]-*n*-hexadecane (Amersham) used as an internal reference. Optical rotations were recorded with a JASCO DIP-370 digital polarimeter.

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(54) Krol, W. J.; Basak, A.; Salowe, S. P.; Townsend, C. A. *J. Am. Chem. Soc.* **1989**, *111*, 7625−7627.
(55) Salowe, S. P.; Marsh, E. N.; Townsend, C. A. *Biochemistry* **1990**, *29*, 6499−6508.
(56) Iwata-Reuyl, D.; Basak, A.; Silverman, L. S.; Engle, C. A.; Townsend, C. A. *J. Nat. Prod.* **1993**, *56*, 1373−1396.
(57) Townsend, C. A.; Basak, A. *Tetrahedron* **1991**, *47*, 2591−2602.
(58) Priestley, N.; Floss, H. G.; Froland, W.; Lipscomb, J. D.; Williams, P. G.; Morimoto, H. *J. Am. Chem. Soc.* **1992**, *114*, 7561−7562.
(59) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923−2925.

Materials. HMPA was refluxed over CaH_2 for 2 h then distilled under reduced pressure. Dry HMPA was used immediately or stored over 4 Å molecular sieves for not more than 2 weeks. CH_2Cl_2 was distilled under Ar from CaH_2. THF was distilled under Ar from sodium benzophenone ketyl. Sodium azide was recrystallized from acetone/H_2O prior to use. All other materials and reagents were used as received unless otherwise stated.

Synthesis of (1*S*,*methyl-S*)-[*N-methyl*-$^2H_1$,^3H]Reticuline. To a buffer (0.87 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 150 nmol of (*S*)-norreticuline were added (*methyl-R*)-AdoMet (30 nmol, 3 μCi, 91% ee) and norreticuline-*N*-methyltransferase (9 pkat). The reaction mixture was incubated at 30 °C for 2 h, and then the pH was adjusted to 9−10 with 0.1 N NaOH. The aqueous mixture was extracted eight times with EtOAc (0.5 mL), and the combined extracts were concentrated under a stream of nitrogen. The residue was further purified by TLC using $CHCl_3$/$(CH_3)_2CO$/Et_2-NH (5:4:1) as the solvent system. The radioactive band due to reticuline was eluted with CH_3OH (20−30 mL) and concentrated under a stream of nitrogen. The reticuline was then dissolved in 0.5 mL of 0.5 mM HCl. Aliquots of the solution were analyzed for radioactivity (1.9 μCi, 63% yield) and chromatographed, and the remainder was evaporated to dryness.

Synthesis of (1*S*,*methyl-R*)-[*N-methyl*-$^2H_1$,^3H]Reticuline. To buffer (0.4 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 150 nmol of (*S*)-norreticuline were added (*methyl-S*)-AdoMet (22 nmol, 3.5 mCi, 86% ee) and norreticuline-*N*-methyltransferase (8 pkat). The reaction mixture was incubated at 30 °C for 3 h, and then the pH was adjusted to 9−10 with 0.1 N NaOH. The reticuline was purified as described above to give 3.2 mCi (93% yield) of the labeled compound.

Degradation of (1*S*,*methyl-S*)-[*N-methyl*-$^2H_1$,^3H]Reticuline. (1*S*)-[*N-methyl*-$^2H_1$,^3H]Reticuline (2.14×10^6 dpm) from (*methyl-R*)-AdoMet was dissolved in 2.5 N H_2SO_4 (10 mL) at 0 °C. The solution was then heated to 80 °C, and $NaNO_2$ (15 mg in 1 mL of H_2O) was slowly added dropwise. The temperature was increased to 110 °C and the reaction mixture stirred for an additional 0.5 h. A solution of 0.05 M $KMnO_4$ was added dropwise, until a purple color persisted, and then the reaction mixture was stirred for 2.5 h. Oxalic acid (12 mg in 1 mL of H_2O) was added to destroy excess $KMnO_4$ and the solution cooled to 0 °C. The solution was adjusted to pH 5 with 2 N NaOH and then refluxed for 12 h. After the pH was readjusted to 1 with 2.5 N H_2SO_4, the solution was lyophilized (bulb to bulb). The solid residue was dissolved in H_2O (3 mL), adjusted to pH 10 with 2 N NaOH, and then lyophilized (bulb to bulb) to give an aqueous solution of methylamine. The methylamine solution was titrated to pH 2 with 1 N HCl, and unlabeled methylamine hydrochloride (4 mg) was added. Lyophilization gave solid methylamine hydrochloride (1.5×10^5 dpm, 7% radiochemical yield).

Formation of Methylamine Ditosylate (24). Methylamine hydrochloride (1.5×10^5 dpm) was dissolved in H_2O (0.1 mL) and the flask cooled to −78 °C. To the cooled flask were added TsCl (86 mg in 1.5 mL of CH_3CN) and 6 N NaOH (0.25 mL), and the mixture was slowly warmed to room temperature. The solution was stirred at room temperature for 3 h and then warmed to 50 °C for an additional 3 h. The cooled (0 °C) solution was neutralized with glacial HOAc and lyophilized to leave a residue which was purified by column chromatography (5 g SiO_2, eluting with $CHCl_3$:hexane, 1:1) to give methylamine ditosylate (1.4×10^5 dpm, 93% radiochemical yield).

Formation of Acetic Acid. Methylamine ditosylate (1.4×10^5 dpm) was dissolved in freshly distilled HMPA (3 mL). KCN (100 mg) was added to the reaction flask, and the flask was connected to a vacuum bridge with a receiver flask containing 1 mL of H_2O. Both flasks were frozen (−78 °C), and the system was evacuated (<1 mmHg) and then closed. The reaction flask was heated (80 °C) with stirring while the receiver flask remained at −78 °C for 3 days. The system was then opened and the receiver flask warmed to room temperature. To the water containing acetonitrile were added 30% H_2O_2 (3 mL) and 5 N NaOH (0.3 mL). The reaction mixture was heated (50 °C) for 7 h, and then the solvent was removed under reduced pressure to leave crude acetamide. The acetamide residue was dissolved in H_2O (1 mL) and cooled to 0 °C, and 5 N H_2SO_4 (1 mL) was added. To the cooled solution was slowly added $NaNO_2$ (0.2 g in 1 mL of H_2O), and the reaction mixture was stirred for 1 h at 0 °C and then for 5 h at room temperature. The solution was adjusted to pH 10 with 5 N NaOH and lyophilized (bulb to bulb). All of the radioactivity remained with the sodium acetate residue. The sodium acetate was dissolved in H_2O (5 mL), and the solution was adjusted to pH 3 with 2 N H_2SO_4 and then distilled in a vacuum bridge. The distillate was adjusted to pH 10 with 0.1 N NaOH and lyophilized to leave sodium acetate (8.3×10^4 dpm, 3.9% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 77 (94% ee of the *S* configuration at the *N*-methyl group).

Degradation of (1*S*,*methyl-R*)-[*N-methyl*-$^2H_1$,^3H]Reticuline. (1*S*)-[*N-methyl*-$^2H_1$,^3H]Reticuline (4.5×10^6 dpm) from (*methyl-S*)-AdoMet was degraded as described above to yield sodium acetate (9×10^4 dpm, 2% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 22 (96% ee of the *R* configuration at the *N*-methyl group).

Incubation of (1*S*,*methyl-R*)- and (1*S*,*methyl-S*)-[*N-methyl*-$^2H_1$,^3H]-Reticuline with BBE and then STOX. Every assay contained in a total volume of 800 μL of 25 mM glycine/NaOH (pH 9.2), 0.25 pkat of BBE, and 4.2 nmol of (5.3 μM) (1*S*,methyl-*R*)- or (1*S*,*methyl-S*)-[*N-methyl*-$^2H_1$,^3H]reticuline (0.42 μCi, 94% ee and 0.67 μCi, 96% ee, respectively). After 120 min of incubation at 37 °C, all the reticuline had been converted to scoulerine (**3**), which was tested by TLC ($CHCl_3$/$(CH_3)_2CO$/Et_2NH, 5:4:1). Then 5 pkat of STOX (12μL) was added, and after a further 3 h of incubation, all the scoulerine had been oxidized to dehydroscoulerine, as determined again by TLC.

Large Scale Preparation of (14*S*)-Scoulerine (3) from (*methyl-R*)-AdoMet for ^3H NMR. Chirally labeled (1*S*,*methyl-R*)-[*N-methyl*-$^2H_1$,^3H]reticuline (0.7 mCi) was synthesized according to the method described above, and then 10 incubations with BBE in 25 mM glycine/NaOH buffer (pH 9.2) were carried out at 37 °C. The reaction was followed by chromatographing samples on TLC ($CHCl_3$/MeOH/NH_3, 68:18:0.6) until reticuline could not be detected anymore. The scoulerine formed was purified by TLC in the same solvent system with a yield of 0.47 mCi. It was repurified immediately prior to NMR spectroscopy to give 153 μCi of material. ^3H NMR (CD_3OD): δ 4.76 (s with ^1H broadband decoupling, d, $J = 15.3$ Hz without ^1H decoupling, 20%, H-8e, δ_{1H} 4.76), 4.29 (br s, 80%, H-8a, δ_{1H} 4.34).

Synthesis of (1*S*,*O*,*N-methyl-S*)-[6-*O*,*N-methyl*-$^2H_1$,^3H]Reticuline. To a buffer (0.55 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 250 nmol of (*S*)-4′-*O*-methylnorlaudanosoline were added (*methyl-R*)-AdoMet (72 nmol, 7.2 μCi, 91% ee) and the two enzymes norlaudanosoline-6-*O*-methyltransferase (9 pkat) and norreticuline-*N*-methyltransferase (25 pkat). The reaction mixture was incubated at 37 °C for 8 h and then purified directly by TLC using $CHCl_3$/$(CH_3)_2CO$/Et_2NH (5:4:1) as the solvent system. The radioactive band due to [6-*O*,*N-methyl*-$^2H_1$,^3H]reticuline was eluted with CH_3OH (20−30 mL) and concentrated under a stream of nitrogen. The [6-*O*,*N-methyl*-$^2H_1$,^3H]reticuline (2 μCi, 28% yield) was then dissolved in 0.5 mL of 0.5 mM HCl.

Synthesis of (1*S*,*O*,*N-methyl-R*)-[6-*O*,*N-methyl*-$^2H_1$,^3H]Reticuline. To a buffer (0.94 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 250 nmol of (*S*)-4′-*O*-methylnorlaudanosoline were added (*methyl-S*)-AdoMet (53 nmol, 8.5 μCi, 86% ee) and the two enzymes norlaudanosoline-6-*O*-methyltransferase (6 pkat) and norreticuline-*N*-methyltransferase (15 pkat). The reaction mixture was incubated at 37 °C for 2.5 h and then purified as described above to give the *methyl-R* isomer of [6-*O*,*N-methyl*-$^2H_1$,^3H]reticuline (3.4 μCi, 41% yield).

Degradation of (1*S*,*O*,*N-methyl-R*)-[6-*O*,*N-methyl*-$^2H_1$,^3H]reticuline. To (1*S*)-[6-*O*,*N-methyl*-$^2H_1$,^3H]reticuline (6.6×10^5 dpm) from (*methyl-S*)-AdoMet were added CH_3CN (1 mL), MeOH (7 μL), and aqueous ceric ammonium nitrate (60 mg in 1.5 mL of H_2O). The reaction mixture was stirred at room temperature for 2 h, benzene (50 mL) was added, and the mixture was distilled to azeotropically remove the methanol formed in the oxidation. To the methanol-containing distillate was added 3,5-dinitrobenzoyl chloride (460 mg) and *N,N*-dimethylaniline (0.26 mL). The solution was then stirred at room temperature for 20 h, H_2O (30 mL) added, and the mixture extracted with benzene. The combined organic extracts were washed with 1 N HCl, followed by saturated $NaHCO_3$ and brine, and then dried (Na_2SO_4). The solvent was removed under reduced pressure and the crude product purified

by column chromatography (10 g SiO_2) using hexane/benzene (1:1) as the solvent to give methyl 3,5-dinitrobenzoate (1.3×10^5 dpm, 19.7% radiochemical yield).

Conversion of Methyl 3,5-Dinitrobenzoate into Acetic Acid. Methyl 3,5-dinitrobenzoate (1.3×10^5 dpm) was converted by the procedure described above for methylamine ditosylate *via* acetonitrile and acetamide (3.2×10^4 dpm, 24.6%) into sodium acetate (3×10^4 dpm, 9% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 25.1 (86% ee of *R* configuration at the 6-*O*-methyl group).

Degradation of (1*S*,*O*,*N*-methyl-*S*)-[6-*O*,*N*-methyl-$^2H_1$,^3H]Reticuline. (1*S*)-[6-*O*,*N*-methyl-$^2H_1$,^3H]Reticuline (1.4×10^6 dpm) from (*methyl*-*R*)-AdoMet was degraded as described above for the (*methyl*-*S*)-AdoMet derived material to give sodium acetate (2.4×10^4 dpm, 4% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 75.6 (88% ee of *S* configuration at the 6-*O*-methyl group).

Synthesis of (1*S*,*methyl*-*S*)-[6-*O*-*methyl*-$^2H_1$,^3H]Reticuline (15). To a buffer (4.2 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 1 mmol of (*S*)-4′-*O*-methyllaudanosoline were added (*methyl*-*R*)-AdoMet (156 nmol, 15.6 μCi, 91% ee) and the enzyme catechol-*O*-methyltransferase (13 pkat). The reaction mixture was incubated at 37 °C for 6 h and then worked up as described earlier to give 13 μCi (83% yield) of the labeled product.

Synthesis of (1*S*,*methyl*-*R*)-[6-*O*-*methyl*-$^2H_1$,^3H]reticuline. To a buffer (2.34 mL) consisting of 50 mM Hepes at pH 7.5 and 10 mM ascorbate and containing 1 μmol of (*S*)-4′-*O*-methyllaudanosoline were added (*methyl*-*S*)-AdoMet (148 nmol, 23.6 μCi, 86% ee) and the enzyme catechol-*O*-methyltransferase (13 pkat). The reaction mixture was incubated at 37 °C for 4.5 h and then worked up as described to give 20 μCi (85% yield) of the labeled compound.

Feeding to Cell Cultures of *Berberis koetineana*. (1*S*,*methyl*-*R*)- (13 μCi, 130 nmol) And (1*S*,*methyl*-*S*)-[6-*O*-*methyl*-$^2H_1$,^3H]reticuline (20 μCi, 125 nmol) were applied to 5−7 g (fresh wt) callus cultures of *B. koetineana*. After 5 days, the calli were extracted twice with 10 mL of MeOH/g of tissue in a homogenizer (Ultra Turrax). The extracts were combined, and the solvent was evaporated. The residues were taken up in 5 mL of MeOH and chromatographed on TLC plates (CH_2Cl_2/MeOH/NH_3, 90:9:1, R_f jatrorrhizine = 0.1). The band corresponding to jatrorrhizine was eluted with 0.2 mM acetic acid in methanol and rechromatographed in $CHCl_3$/MeOH/NH_3, 68:18:0.6 (R_f jatrorrhizine = 0.3).

Degradation of Jatrorrhizine. [2-*O*-*methyl*-$^2H_1$,^3H]Jatrorrhizine (3.5×10^5 dpm) from the (1*S*,*methyl*-*S*)-[6-*O*-*methyl*-$^2H_1$,^3H]reticuline feeding to callus cultures of *B. koetineana* was degraded by the same procedure described above for [6-*O*-*methyl*-$^2H_1$,^3H]reticuline to give sodium acetate (1.6×10^4 dpm, 5% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 55.4 (19% ee of *S* configuration at the 2-*O*-methyl group).

Similarly, [2-*O*-*methyl*-$^2H_1$,^3H]jatrorrhizine (4.8×10^5 dpm) from the (1*S*,*methyl*-*R*)-[6-*O*-*methyl*-$^2H_1$,^3H]reticuline-feeding experiment gave sodium acetate (2.4×10^4 dpm, 5% overall radiochemical yield). Chirality analysis of the acetic acid gave an *F* value of 44.2 (20% ee of *R* configuration at the 2-*O*-methyl group).

Synthesis of 3,5-Dimethoxy[7-$^2H_2$]benzyl Alcohol. To a cooled (0 °C) solution of methyl 3,5-dimethoxybenzoate (5.88 g, 30 mmol) in dry THF (50 mL) under an Ar atmosphere was added $LiAlD_4$ (1.0 g, 23.8 mmol) in portions over a 30 min period. The reaction mixture was stirred at 30 °C for 5 h and then cooled (0 °C). Excess $LiAlD_4$ was carefully destroyed with 6 N H_2SO_4. CH_2Cl_2 (50 mL) was added and additional 6 N H_2SO_4 used to dissolve any Al salts. The aqueous phase was separated and extracted with CH_2Cl_2 (2 × 25 mL). The combined organic extract was washed sequentially with saturated $NaHCO_3$, H_2O, and brine and then dried (Na_2SO_4). The solution was filtered and concentrated. The residue was recrystallized from Et_2O/pentane to afford colorless needles (4.5 g, 88% yield) with mp = 49−50 °C (lit.[60] mp 48−48.5 °C). ^1H NMR ($CDCl_3$): δ 6.49 (d, 2H, J = 2.34), 6.36 (t, 1H, J = 2.34), 3.77 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 160.93, 143.34, 104.52, 99.60, 65.26 ($^{13}C^2H_2$, deuterium-decoupled), 55.29.

(60) Ridley, D. D.; Ritchie, E.; Taylor, W. C. *Aust. J. Chem.* **1968**, *21*, 2979−2988.

Synthesis of 3,5-Dimethoxy[7-$^2H_1$]benzaldehyde (18). To a solution of 3,5-dimethoxy[7-$^2H_2$]benzyl alcohol (7.2 g, 42 mmol) in CH_2Cl_2 (150 mL) was added pyridinium chlorochromate (PCC) (8.0 g, 37 mmol), and the mixture was stirred vigorously for 30 min. An additional portion of PCC (12 g, 56 mmol) was added and the reaction stirred for 6 h at room temperature. The reaction mixture was then poured into wet ether (500 mL), and the Cr salts were washed with additional ether (3 × 100 mL). The combined ether solution was filtered through Celite and concentrated. The residue was purified by flash chromatography (SiO_2, 2 × 10 cm column), eluting with benzene. The product was then recrystallized from ether/pentane to afford colorless cubes (5.3 g, 75% yield) with mp = 46−47 °C (lit.[60] mp 46.5−47 °C). ^1H NMR ($CDCl_3$): δ 6.99 (d, 2H, J = 2.34), 6.69 (t, 2H, J = 2.34), 3.80 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 191.91 ($^{13}C^2H$, deuterium-decoupled), 161.23, 138.39, 107.15, 107.09, 55.61.

Synthesis of (7*R*)-3,5-Dimethoxy[7-$^2H_1$]benzyl Alcohol (19). To a solution of 9-BBN (3.11 g, 25 mmol) in THF (50 mL) under Ar was added (1*S*)-(−)-α-pinene (4.09 g, 30 mmol), and the mixture was refluxed for 6.5 h. It was then cooled (0 °C), and 3,5-dimethoxy[7-^2H]benzaldehyde (2.51 g, 15 mmol) was added. The reaction mixture was stirred at room temperature for 15 h and then refluxed for 1 h. Excess reducing agent was destroyed by the addition of acetaldehyde (0.5 mL), and the reaction mixture was stirred for an additional 15 min at room temperature. The solvent was evaporated and α-pinene pumped off in a vacuum (0.1 mmHg, 40 °C). To the residue were added ether (15 mL) and ethanolamine (0.75 mL), and the mixture was stirred for 30 min. The solution was filtered and the precipitate washed with ether. The combined organic solution was washed with brine and dried (Na_2SO_4), and the solvent was removed to leave a residue that was further purified by flash chromatography (EtOAc/petroleum ether, 1:1) and recrystallization from ether/pentane to afford colorless needles (1.90 g, 75% yield) of mp 45−46 °C. ^1H NMR ($CDCl_3$): δ 6.49 (d, 2H, J = 2.34), 6.36 (t, 1H, J = 2.34), 3.77 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 160.93, 143.34, 104.52, 99.60, 65.26 ($^{13}C^2H^1H$, deuterium-decoupled), 55.29.

Synthesis of (7*R*)-3,5-Dimethoxy[7-$^2H_1$]benzyl Tosylate (20). To a suspension of NaH in mineral oil (87 mg, 60% oil dispersion, 2.1 mmol) in 8 mL of dry THF was added **16** (333 mg, 2.0 mmol) under an Ar atmosphere. The reaction mixture was stirred at 50 °C for 15 h and then cooled to 0 °C. *p*-Toluenesulfonyl chloride (TsCl, 381 mg, 2.0 mmol) was dissolved in 2 mL of dry THF and added dropwise to the reaction mixture. The mixture was stirred at 0 °C for 1 h, warmed to room temperature, and stirred for an additional 2 h. Insoluble material was filtered off and washed with a small amount of THF. The combined THF solution was concentrated to <10 mL and cooled to −60 °C to give colorless needles of the tosylate (330 mg, 51.1% yield).

Synthesis of (7*R*)-3,5-Dimethoxy[7-$^2H_1$,$^3H_1$]toluene (21). (7*R*)-3,5-Dimethoxy[7-$^2H_1$]benzyl tosylate (161 mg, 0.5 mmol) was dissolved in 1 mL of THF and added to a solution of carrier-free $LiEt_3BT$ (1 mmol, 29 Ci in 2 mL of hexane/THF solution containing 170 μL of TMEDA)[41] at 0 °C. The solution was warmed to room temperature and stirred for 1 h. Excess $LiEt_3BT$ was destroyed by addition of 1 mL of methanol. The solvent was removed under reduced pressure and the residue dissolved in 1 mL of HCl/methanol solution. This solution was concentrated, and the residue was dissolved in EtOAc and then washed with 0.1 N KOH solution. The organic layer was separated and the aqueous phase washed with EtOAc. The combined organic solution was dried (Na_2SO_4) and concentrated under a stream of nitrogen. The residue was dissolved in Et_2O and passed through a short SiO_2 column. The eluent was monitored for radioactivity, and fractions containing the product were combined and concentrated. Total radioactivity = 9.4 Ci. ^1H-coupled ^3H NMR (C_6D_6): δ 2.10 (d, J_{H-T} = 14.7).

Synthesis of (*R*)-[2-$^2H_1$,^3H]Acetic Acid. The chiral acetic acid was prepared from **17** (4 Ci) by ozonolysis as previously described[40] to yield (*R*)-[2-^2H,^3H]acetic acid (2.3 Ci) as the sodium salt. ^3H NMR (D_2O): δ 1.98 (CH_2T, 7.5%), 1.97 (CHDT, 92.5%).

Synthesis of (*methyl*-*R*)-[*N*-*methyl*-$^2H_1$,^3H]Methylamine (23). Sodium (*R*)-[2-$^2H_1$,^3H]acetate (1.45 Ci) was dissolved in 4 mL of H_2O and the solution added to a reaction flask. The water was removed by lyophilization and the flask cooled to 0 °C. The residue in the cooled

flask was dissolved in 2 mL of 100% H_2SO_4 (prepared by adding 2 mL concentrated H_2SO_4 to 1 mL of fuming H_2SO_4). The flask was heated to 50 °C until all of the solid had dissolved. While the temperature was kept at 45−50 °C, NaN_3 (360 mg, recrystallized from water/acetone and dried in an Abderhalden drying pistol with P_2O_5 at 60 °C) was added in a few small portions. Bubbling occurs due to evolution of N_2 and CO_2. After addition was complete, the oil bath temperature was increased to 80 °C for 1 h. The reaction mixture was cooled to 0 °C, and 2 mL of H_2O was added carefully. The solution was then basified (pH 14) with 5 N NaOH. A stream of N_2 was passed through the stirred solution and then through two spiral traps containing 2 N HCl. The reaction flask was heated to 90 °C for 2 h. Aliquots counted from both traps and the reaction flask indicated that the majority of the radioactivity was present in the traps. The contents of the acid traps were combined, and the solvent was removed under reduced pressure to leave a residue of methylamine hydrochloride, which was used directly to prepare the monotosylate.

Synthesis of (*methyl-R*)-[*N-methyl*-$^2H_1$,^3H]Methyltosylamine. The methylamine hydrochloride residue from above was dissolved in H_2O (5 mL), and CH_2Cl_2 (15 mL) was added. TsCl (205 mg, 1.07 mmol) was added, and the reaction mixture was stirred until the solid had completely dissolved. The solution was then cooled to −78 °C with stirring, and NaOH (190 mg, 4.75 mmol), dissolved in a small volume of water, was added to the reaction flask. The flask was sealed with a rubber septum, and the contents were slowly warmed to room temperature and stirred for 18 h. The reaction mixture was then added to a separatory funnel containing brine (20 mL) and CH_2Cl_2 (20 mL). The organic layer was separated and the aqueous layer extracted twice with CH_2Cl_2 (15 mL). The combined organic solution was dried ($MgSO_4$) and the solvent removed to leave a solid. TLC showed that it consisted of methylamine monotosylate and TsCl. The crude product was used directly in the conversion to the ditosylate.

Synthesis of (*methyl-R*)-[*N-methyl*-$^2H_1$,^3H]Methylamine Ditosylate (24). A stirred slurry of NaH (250 mg, 60% oil dispersion) in 25 mL of freshly distilled (from CaH_2) CH_2Cl_2 was cooled to −78 °C under an atmosphere of dry nitrogen, and the crude monotosylate from above, dissolved in 2 mL of CH_2Cl_2, was added *via* a syringe. The reaction flask was then slowly warmed to room temperature over 20 min (H_2 gas evolves) and then cooled back to −78 °C. TsCl (250 mg, 1.3 mmol) was dissolved in CH_2Cl_2 and added to the reaction flask by a syringe. After stirring for 10 min at −78 °C, the cooling bath was removed and the reaction mixture stirred for 16 h at room temperature. The contents of the reaction flask were then added to a separatory funnel containing brine (20 mL) and CH_2Cl_2 (20 mL). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extract was dried ($MgSO_4$), and the solvent was removed under reduced pressure to leave a solid. The crude product was dissolved in a small amount of $CHCl_3$ and spotted on four preparative TLC plates (20 × 20 cm, 2 mm SiO_2). The plates were developed using $CHCl_3$ as solvent. The zone corresponding to the ditosylate (R_f = 0.42) was marked under UV light and scraped off the plate, and the product was extracted from the silica with $CHCl_3$. The solution was concentrated under reduced pressure to a small volume (5 mL) and the remaining solvent evaporated under a stream of dry nitrogen to leave *(R)*-[*methyl*-$^2H_1$,^3H]methylamine *N,N*-ditosylate (285 mg, 0.88 mmol, 662 mCi, specific radioactivity = 755 mCi/mmol). ^1H NMR ($CDCl_3$): δ 7.86 (d, 4H, ArH), 7.34 (d, 4H, ArH), 3.25 (s, 3H, NCH_3), 2.43 (s, 6H, $ArCH_3$). ^3H NMR ($CDCl_3$): δ 3.25 (CH_2T, 15%), 3.23 (CHDT, 85%).

Synthesis of (*methyl-S*)-[*methyl*-$^2H_1$,^3H]Methionine (26). *S*-Benzyl-L-homocysteine (201 mg, 0.893 mmol) was dissolved in liquid NH_3 (40 mL) at −78 °C. To the stirred solution was added sodium metal in small pieces until a dark blue color was maintained for 30 min. The cold bath was then removed and the liquid NH_3 allowed to evaporate slowly under an Ar atmosphere. When ~2 mL of NH_3 remained, freshly distilled HMPA (10 mL) was added to the reaction flask through a septum. The remainder of the NH_3 was evaporated with gentle warming. A solution of **24** (285 mg, 0.876 mmol, 1.46 × 10^{12} dpm, 755 mCi/mmol) in dry HMPA (5 mL) was added to the reaction flask *via* a syringe. The reaction flask was heated to 90 °C for 18 h with stirring. The reaction mixture was then adjusted to pH 5 with 2 N HCl. The entire contents of the flask were poured onto an ion-exchange column (Dowex 50 × 8, 200 mesh, H^+ form, 2 × 30 cm). The column was washed with 125 mL of H_2O to remove HMPA. The methionine was eluted with 5% NH_4OH solution. Small aliquots of the collected fractions were spotted on filter paper and tested with ninhydrin spray. All fractions that tested positive for amino acids were analyzed by TLC (silica gel, butanol/HOAc/H_2O, 4:1:1). Each fraction showed methionine as the major spot (R_f = 0.28) with a small amount of base-line impurity, probably homocysteine. These fractions were combined and concentrated to give crude methionine (240.3 mCi), which was dried under vacuum (0.2 mmHg) and dissolved in a minimal amount of hot glacial acetic acid. Benzene was added until the solution became cloudy. Crystals formed when the solution was kept overnight at room temperature. The mother liquor was removed through a capillary tube, and the crystals were carefully washed with ether and dried under vacuum. Total radioactivity of the recrystallized methionine = 147 mCi, specific radioactivity = 751 mCi/mmol. ^3H NMR (D_2O): δ 2.06 (CH_2T, 9%), 2.05 (CHDT, 91%). Degradation[27] gave acetic acid with an *F* value of 34, indicating 55% ee *S* configuration of the methionine methyl group.

Formation of (*methyl-S*)-[*methyl*-$^2H_1$,^3H]Adenosylmethionine (AdoMet). *S*-Adenosylmethionine synthetase was prepared from *Escherichia coli* DM 25 pk8, immobilized on silylated glass, and used to convert L-methionine quantitatively into AdoMet as previously described.[46]

Synthesis of (*methyl-R*)-(±)-[3-*O-methyl*-$^2H_1$,^3H]tetrahydrocolumbamine (29). Incubations contained labeled (*methyl-S*)-AdoMet (11.75 μmol, 8.8 mCi), desmethyleneberberine (120 μmol), ascorbate (500 μmol), potassium phosphate buffer (1 mmol, pH 7), and porcine catechol-*O*-methyltransferase (50 pkat) in a total volume of 10 mL. The reaction mixtures were incubated for 8 h at 30 °C. The combined reaction mixtures from 10 incubations were chromatographed using XAD-2 adsorbent resin. The alkaloids were eluted with methanol, reduced with an excess of $NaBH_4$, and purified by TLC (toluene/EtOAc/Et_2NH, 7:2:1). The radioactive band corresponding to tetrahydrocolumbamine (R_f = 0.5) was eluted with CH_3OH and the solvent evaporated under a steam of nitrogen. Yield: 51.75 mCi = 58.8%.

Feeding to *T. tuberosum* Cell Cultures. (14*RS*,*methyl-R*)-[3-*O-methyl*-$^2H_1$,^3H]Tetrahydrocolumbamine (51.75 mCi) was dissolved in DMSO and fed to five separate flasks containing 4-day-old cell cultures of *T. tuberosum* (white cell line) in 150 mL of medium.[61] After 60 h, the cells were harvested (35 g fresh wt) and extracted with ethanol. The combined ethanol extracts were concentrated, and the berberine was purified by TLC ($CHCl_3/CH_3OH/NH_3$, 70:12:1, R_f berberine = 0.4). The radioactive band corresponding to berberine was removed, extracted with CH_3OH, and rechromatographed in the same solvent system to yield berberine (2.75 mCi, 5.3% radiochemical yield).

Formation of (14*RS*)-[*methylenedioxy*-$^2H_1$,^3H]Canadine (31). The crude labeled berberine (2.75 mCi) was dissolved in MeOH (25 mL), and $NaBH_4$ (100 mg, 2.64 mmol) was added slowly in small portions. The reaction mixture was stirred for 30 min at room temperature, and then the solvent was removed under reduced pressure. The residue was dissolved in water and extracted with EtOAc (4 × 30 mL). The combined organic extract was dried (Na_2SO_4) and concentrated to leave 75 mg of solid (total radioactivity = 2.74 mCi). The crude product was purified by flash chromatography (SiO_2, 2.0 × 12 cm) using $CHCl_3$, followed by $CHCl_3$/MeOH/NH_3 (185:15:0.1), and finally $CHCl_3$/MeOH (1:1). $CHCl_3$/MeOH/NH_3 (185:15:0.1) eluted the labeled (±)-canadine (1.05 mCi, 38% radiochemical yield from crude berberine). ^3H NMR ($CDCl_3$): δ 5.97 (s, CHT, 15−20% of the total integrated area), 5.94 (s, CDT). ^1H NMR ($CDCl_3$): δ 6.80 (d, 1H, J = 8.4, H-11), 6.74 (d, 1H, J = 8.4, H-12), 6.70 (s, 1H, H-1), 6.56 (s, 1H, H-4), 5.97 (s, 2H, OCH_2O), 4.20 (d, 1H, J = 15.8, H-8e), 3.82 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.50 (m, 2H, H-8a + H-14), 3.15 (m, 3H, H-13e + H-6 + H-5), 2.80 (dd, 1H, ^3J = 11.8, ^2J = 15.8, H-13a), 2.60 (m, 2H, H-6 + H-5).

Resolution of Labeled (14*R*)-(+)- and (14*S*)-(−)-Canadine (32, 34). Labeled (14*R,S*)-canadine (1.05 mCi) was dissolved in CH_2Cl_2 (0.5 mL) and diluted to 1.6 mL with hexane/i-PrOH/Et_2NH (80:20:0.1). The mixture was then resolved by HPLC on a Chiralcel OD (1 × 25 cm) column using hexane/i-PrOH/Et_2NH (80:20:0.1) as the mobile

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phase. The following parameters were used: injection size = 0.8 mL, flow rate = 2.4 mL/min, UV detection at 254 nm. (14*R*)-(+)-Canadine had a retention time of 14 min (450 μCi, $[\alpha]_D = +188°$, $c = 0.25$). ^{3}H NMR ($CDCl_3$): δ 5.97 (CHT), 5.94 (CDT). ^{1}H NMR ($CDCl_3$): δ 6.80 (d, 1H, $J = 8.4$, H-11), 6.74 (d, 1H, $J = 8.4$, H-12), 6.70 (s, 1H, H-1), 6.56 (s, 1H, H-4), 5.97 (s, 2H, OCH_2O), 4.20 (d, 1H, $J = 15.8$, H-8e), 3.82 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.50 (m, 2H, H-8a + H-14), 3.15 (m, 3H, H-13e + H-6 + H-5), 2.80 (dd, 1H, $^3J = 11.8$, $^2J = 15.8$, H-13a), 2.60 (m, 2H, H-6 + H-5). (14*S*)-(−)-Canadine had a retention time of 44 min (485 μCi, $[\alpha]_D = -192°$, $c = 0.25$). ^{3}H NMR ($CDCl_3$): δ 5.97 (CHT), 5.94 (CDT). ^{1}H NMR ($CDCl_3$): δ 6.80 (d, 1H, $J = 8.4$, H-11), 6.74 (d, 1H, $J = 8.4$, H-12), 6.70 (s, 1H, H-1), 6.56 (s, 1H, H-4), 5.97 (s, 2H, OCH_2O), 4.20 (d, 1H, $J = 15.8$, H-8e), 3.82 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.50 (m, 2H, H-8a + H-14), 3.15 (m, 3H, H-13e + H-6 + H-5), 2.80 (dd, 1H, $^3J = 11.8$, $^2J = 15.8$, H-13a), 2.60 (m, 2H, H-6 + H-5). Fractions were collected at 2 min intervals. Radioactivity assay of each fraction showed that all of the sample's radioactivity eluted with the two enantiomers.

Derivatization of (14*R*)-(+)-[2-^{2}H$_1$,^{3}H]Canadine. To a flask containing *p*-nitrobenzyl bromide (17.5 mg, 0.08 mmol) were added unlabeled (14*R*)-(+)-canadine (13.2 mg, 0.04 mmol) and a solution of (14*R*)-(+)-[2-^{2}H$_1$,^{3}H]canadine (300 μCi) in CH_2Cl_2 (3 mL). The reaction mixture was stirred under Ar for 3 days. The product was purified by flash chromatography (2 × 17 cm), eluting with $CHCl_3$ to remove excess *p*-nitrobenzyl bromide and then changing to $CHCl_3$/CH_3OH (10:1) to elute (14*R*)-canadine derivative **33** (20 mg, 270 μCi, 90% radiochemical yield). $[\alpha]_D = +48.5°$ ($c = 1.0$). ^{3}H NMR ($CDCl_3$): δ 6.01, 5.99, 5.97 (0.27:1.81:1.0 integration ratio). ^{1}H NMR ($CDCl_3$): δ 8.27 (d, 2H, $J = 9.8$), 7.95 (d, 2H, $J = 9.8$), 6.86 (d, 2H), 6.76 (d, 2H), 6.03 (d, 1H), 5.97 (d, 1H), 5.74 (d, 1H), 5.32 (m, 1H), 5.23 (d, 1H), 4.97 (d, 1H), 4.85 (d, 1H), 4.47 (m, 1H), 3.88 (s, 3H), 3.82 (s, 3H), 3.53 (m, 3H), 3.18 (m, 2H).

Derivatization of (14*S*)-(−)-[2-^{2}H$_1$,^{3}H]Canadine. In the same manner, (14*S*)-(−)-[2-^{2}H$_1$,^{3}H]canadine (13.9 mg, 0.04 mmol, 300 μCi) was reacted with *p*-nitrobenzyl bromide to give (14*S*)-canadine derivative **35** (19 mg, 264 μCi, 88% radiochemical yield). $[\alpha]_D = -48.8°$ ($c = 1.0$). ^{3}H NMR ($CDCl_3$): δ 6.01, 5.99, 5.97 (0.28:0.98:1.0 integration ratio). ^{1}H NMR ($CDCl_3$): δ 8.27 (d, 2H, $J = 9.8$), 7.95 (d, 2H, $J = 9.8$), 6.86 (d, 2H), 6.76 (d, 2H), 6.03 (d, 1H), 5.97 (d, 1H), 5.74 (d, 1H), 5.32 (m, 1H), 5.23 (d, 1H), 4.97 (d, 1H), 4.85 (d, 1H), 4.47 (m, 1H), 3.88 (s, 3H), 3.82 (s, 3H), 3.53 (m, 3H), 3.18 (m, 2H).

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