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BISBENZYLISOQUINOLINE BIOSYNTHESIS IN *BERBERIS STOLONIFERA* CELL CULTURES*

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Key Word Index—*Berberis stolonifera*; Berberidaceae; cell cultures; biosynthesis; bisbenzylisoquinoline alkaloids; 2-norberbamunine; berbamunine; guattegaumerine; aromoline; berbamine; isotetrandrine.

Abstract—Bisbenzylisoquinoline alkaloid-producing cell cultures of *Berberis stolonifera* were fed with ^{14}C-labelled tyrosine, tyramine, and several chiral 1-benzyl-1,2,3,4-tetrahydroisoquinolines. Berbamunine subsequently isolated from these cultures showed significantly higher incorporation than 2-norberbamunine, berbamine, aromoline or isotetrandrine, suggesting that it is the first dimer formed in these cells. L-Tyrosine labelled the isoquinoline and benzyl moieties of berbamunine is *ca* equal ratios (1.2:1). Tyramine was almost exclusively incorporated into the isoquinoline portion of this dimer. (*R*)-*N*-Methylcoclaurine gave the highest relative incorporations into bisbenzylisoquinolines (29% into berbamunine), followed by (*R*)-coclaurine (10%), (*S*)-coclaurine (5.2%), and (*S*)-*N*-methylcoclaurine (2.4%). As both (*S*)-coclaurine and (*S*)-*N*-methylcoclaurine are well incorporated into the protoberberine fraction (21 and 54%, respectively), these results indicate that the dimerization pathway and the C-3′-hydroxylation leading to reticuline are competing for these substrates. Feeding experiments with (1-^{13}C)-(*R*)- and (*S*)-coclaurine and NMR studies of the resulting alkaloids confirmed the biosynthetic route to berbamunine, and also showed that the (*R*) isomer is incorporated in similar isotopic excess into both halves of the (*R*, *R*) dimer guattegaumerine which had not been found previously in *Berberis* species. No racemization of (*S*)- to (*R*)-coclaurine (or its derivatives) or vice versa occurs in this tissue.

INTRODUCTION

According to biogenetic theory, bisbenzylisoquinoline alkaloids (BBIQ's) are products of dimerization of 6,7,4′-trioxygenated benzyltetrahydroisoquinolines (BIQ's) [1] in which phenyl ether and aryl–aryl bonds are formed by oxidative phenol coupling [2]. The last 20 years have seen the publication of a number of papers on the expected *in vivo* incorporation of labelled coclaurines and *N*-methylcoclaurines into BBIQ's [3–10]. The results of these investigations indicate that both enantiomers of the secondary amine coclaurine and of its *N*-methyl derivative may act as BBIQ precursors, and that their incorporation appears to be stereospecific.

The *N*-methylcoclaurines arise in nature by non-stereospecific methylation of the corresponding coclaurines, catalysed by *S*-adenosylmethionine: (*R*),(*S*)-norreticuline-*N*-methyltransferase [11]. Coclaurine, in turn, is a methylation product of norcoclaurine (demethylcoclaurine, higenamine) catalysed by *S*-adenosylmethionine: (*R*),(*S*)-norlaudanosoline-6-*O*-methyltransferase [12], which in the light of our recent biogenetic findings [13] should now be renamed, taking into account its physiological substrates, as *S*-adenosylmethionine:(*R*),(*S*)-norcoclaurine-6-*O*-methyltransferase. Whether the trioxygenated BIQ's of the (*R*) series arise independently or are derived from one or more of their (*S*) counterparts is still unknown. (*S*)-Norcoclaurine, on the other hand, is produced by (*S*)-norcoclaurine synthase [formerly (*S*)-norlaudanosoline synthase] by a stereospecific Pictet–Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde [14]. The origin of these early precursors of the BBIQ's has been discussed recently [15].

Cell cultures of *Berberis stolonifera* are rich sources of BBIQ's. Calli of this species contain about 1% of 2-norberbamunine (**1**) (dry weight basis), slightly less berbamunine (**2a**), and smaller but still quite considerable concentrations of aromoline (**5a**), berbamine (**6b**) and isotetrandrine (**6c**) [16]. In suspension cultures of our *B. stolonifera* V29 cell line berbamunine is by far the major BBIQ, attaining concentrations in excess of 250 mg/l medium. All these dimers are biogenetically interrelated [3] and possess the 1-(*R*),1′-(*S*) absolute configuration. In the present work we have fed *B. stolonifera* V29 callus and suspension cultures with ^{14}C-labelled tyrosine, tyramine, and (*R*)- and (*S*)-BIQ's and studied their incorporation into BBIQ's in an attempt to improve our understanding of the biosynthesis of this important class of alkaloids.

RESULTS

Feeding experiments with tyrosine

L-Tyrosine is a well known precursor of both the isoquinoline and the benzyl moieties of monomeric BIQ's [17] and is therefore expected to label the 'upper' (iso-

*Dedicated to the memory of Tony Swain.

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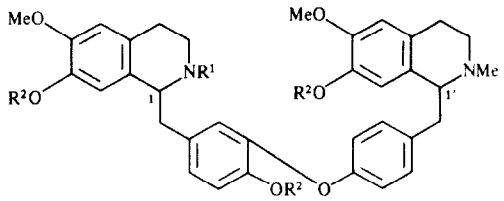
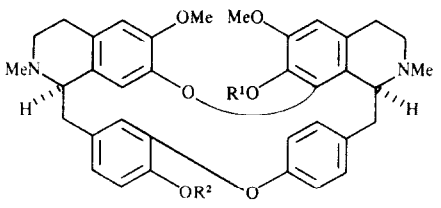
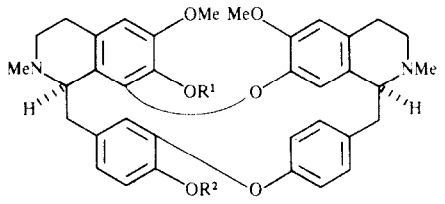


1 2-Norberbamunine $R^1 = R^2 = H$; C-1: (*R* = β), C-1': (*S* = α)
2a Berbamunine $R^1 = Me$, $R^2 = H$; C-1: (*R*), C-1': (*S*)
2b *O,O',O''*-Trimethylberbamunine $R^1 = R^2 = Me$; C-1: (*R*), C-1': (*S*)
2c Guattegaumerine $R^1 = Me$, $R^2 = H$; C-1: (*R*), C-1': (*R*)



5a Aromoline $R^1 = R^2 = H$
5b Oxyacanthine $R^1 = Me$, $R^2 = H$
5c Obaberine $R^1 = R^2 = Me$



6a Obamegine $R^1 = R^2 = H$
6b Berbamine $R^1 = Me$, $R^2 = H$
6c Isotetrandrine $R^1 = R^2 = Me$

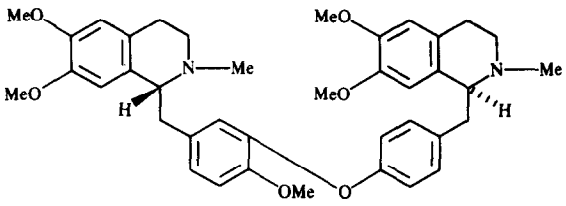
quinoline) and 'lower' (benzyl) halves of tail-to-tail coupled **BBIQ**. Although radioactive L-tyrosine has been fed to plants of *Stephania japonica* and *Tiliacora racemosa* and to cut branches of *Cocculus laurifolius* synthesizing **BBIQ**'s [3, 4, 6, 8], the distribution of the tracer was not determined. Five days after applying (U-^{14}C)tyrosine to calli and suspension cultures of *B. stolonifera* V29, all five identified **BBIQ**'s were labelled as shown in Table 1. In the latter case it is noteworthy that 9% of the applied radioactivity was recovered in berbamunine (*ca* 2% for the calli), underscoring one of the advantages of cell cultures over whole differentiated plants or plant organs (cf. 0.17% into epistephanine [3], 0.09% into tiliacorine, 0.06% into tiliacorinine [6], 0.10% into cocsulin [4] and 0.09% into oxyacanthine, **5b** [8]).

Labelled berbamunine from callus cultures was diluted with carrier and purified to constant specific activity through its tri-*O*-acetyl derivative and *O,O',O''*-trimethylberbamunine (**2b**). The latter was oxidized with cerium (IV) ammonium nitrate (CAN) and worked-up [18] to afford 6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (**3**) and 3-(4-hydroxymethylphenoxy)-4-methoxybenzyl alcohol (**4**) (Scheme 1). The specific activities of these compounds, shown in Table 2, clearly indicate that tyrosine is incorporated into the isoquinoline and benzyl moieties of berbamunine in a ratio of *ca* 1.2 to 1.

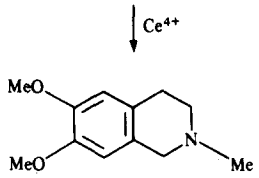
Table 1. Distribution of radioactivity in the bisbenzylisoquinoline alkaloids of *B. stolonifera* V29 cultures after feeding with (U-^{14}C)tyrosine (2.5 μCi, 4.96 nmol)

Culture	Alkaloid	Amount isolated (μmol)	Rel. incorporation (%)*	Sp. act. (dpm./μmol)
Callus	2-Norberbamunine	1.33	0.96	23×10^3
	Berbamunine	2.56	3.54	43×10^3
	Aromoline	0.49	0.17	11×10^3
	Berbamine	0.54	0.18	11×10^3
	Isotetrandrine	0.24	0.03	3.4×10^3
Suspension	2-Norberbamunine	1.41	1.22	17×10^3
	Berbamunine	18.9	25.9	26×10^3
	Aromoline	1.18	1.38	23×10^3
	Berbamine	3.04	3.71	24×10^3
	Isotetrandrine	0.54	0.29	10×10^3

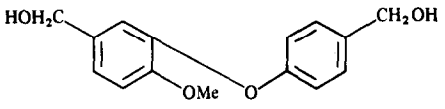
* Percentage of the radioactivity of the crude methanol extract of the tissue.



2b *O,O',O''*-Trimethylberbamunine



3 6,7-Dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline



4 3-(4-Hydroxymethylphenoxy)-4-methoxybenzyl alcohol

Scheme 1. Oxidation of the *O*, *O'*, *O''*-trimethyl derivative of berbamunine with cerium (IV) ammonium nitrate (CAN).

Feeding experiments with tyramine

It has been reported over and over again that dopamine, which is either derived by hydroxylation of tyramine or by decarboxylation of DOPA, is only incorporated into the isoquinoline portion of monomeric BIQ's [17]. Recent work has shown, however, that the incorporation of tyramine into the 'benzyl' moiety of jatrorrhizine in calli of *B. canadensis*, though considerably less than the corresponding value for the 'isoquinoline' portion, is also quite significant, and that tyramine leads to the labelling of both moieties in a 3:1 ratio [15]. (7-^{14}C)Tyramine was applied to calli and suspension cultures of *B-stolonifera* V29 which were harvested and worked-up after five days. The BBIQ's were labelled as shown in Table 3. Relative incorporations were similar to those obtained after feeding radioactive tyrosine.

Labelled berbamunine and berbamine from calli and berbamunine from suspension cultures were purified and derivatized to constant specific activities and the *O*-methylated derivatives were oxidized with CAN. In both types of cultures and for both alkaloids, almost all the radioactivity from tyramine was concentrated in the 'upper' half of the dimers, while the 'lower' half of each alkaloid isolate contained not more than 5% of the tracer (Table 2). It seems therefore that calli of *B. canadensis* may be exceptional in their greater ability to utilize tyramine in the construction of the benzyl portion of isoquinoline alkaloids [15]. The more common low or even insignificant incorporation of tyramine into the 'lower' half of these products is probably a consequence of weak amine oxidase activity in plant tissues or in metabolic compartments associated with isoquinoline alkaloid biosynthesis. Such low amine oxidase activities have been demonstrated for four *Berberis* and one *Tinospora* (Menispermaceae) species [15].

Another isolate of labelled berbamunine from *B. stolonifera* V29 calli was purified and derivatized as before, and reduced with sodium in liquid ammonia [19] to give (*R*)-4'-*O*-methylarmepavine (specific activity: 9800 dpm/μmol) and (*S*)-armepavine (specific activity: 6200 dpm/μmol). The specific activities of these products showed that both BIQ halves of berbamunine were strongly labelled, although the incorporation of tyramine into the (*R*) half was greater by 50%. This preference is

Table 2. Distribution of radioactivity in the CAN oxidation products of the methyl ethers of berbamunine and berbamine from *B. stolonifera* V29 callus cultures fed with (U-^{14}C)tyrosine or (7-^{14}C)tyramine

Precursor (dpm/lmol)	Compound	Amount isolated (μmol)	Sp. act. (dpm/μmol)
(U-^{14}C)Tyrosine	Berbamunine*	4.0	4.0×10^3
	'upper' half†	0.71	2.1×10^3
	'lower' half‡	0.47	1.7×10^3
(7-^{14}C)Tyramine	Berbamunine*	7.66	4.7×10^3
	'upper' half†	1.92	4.7×10^3
	'lower' half‡	1.21	0.04×10^3
(7-^{14}C)Tyramine	Berbamine§	3.52	1.2×10^3
	'upper' half‖	0.76	1.2×10^3
	'lower' half‡	0.70	0.06×10^3

* As its *O,O',O''*-trimethyl derivative.

† 6,7-Dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline.

‡ 3-(4-Hydroxymethylphenoxy)-4-methoxybenzyl alcohol.

§ As isotetrandrine.

‖ 6,7-Dimethoxy-8-(6-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline-7-oxy)-2-methyl-1,2,3,4-tetrahydroisoquinoline.

Table 3. Distribution of radioactivity in the bisbenzylisoquinoline alkaloids of *B. stolonifera* V29 cultures after feeding with (7-^{14}C)tyramine (2.5 μCi, 45 nmol)

Culture	Alkaloid	Amount isolated (μmol)	Rel. incorporation (%)*	Sp. act. (dpm/μmol)
Callus	2-Norberbamunine	4.28	1.57	19×10^3
	Berbamunine	8.90	3.50	20×10^3
	Aromoline	3.04	0.49	8.5×10^3
	Berbamine	3.50	0.51	7.7×10^3
	Isotetrandrine	1.64	0.06	1.9×10^3
Suspension	2-Norberbamunine	1.00	0.96	24×10^3
	Berbamunine	11.2	20.0	45×10^3
	Aromoline	0.75	0.91	31×10^3
	Berbamine	1.42	1.82	32×10^3
	Isotetrandrine	0.50	0.25	13×10^3

* See Table 1, note*.

discussed below together with the results obtained after feeding labelled BIQ's.

Feeding experiments with chiral BIQ's

Previous tracer studies using chiral coclaurines and *N*-methylcoclaurines suggested that the incorporation of these precursors into BBIQ's occurs stereospecifically [3–10]. The assumption that this is at least an approximation to reality was basic to the elucidation of the absolute configurations of tiliacorine and tiliacorinine [6]. These older papers also give the impression that *N*-methylcoclaurine is a better precursor of BBIQ's than coclaurine. The low incorporations and the resulting imprecisions in the published data, however, leave much room for doubt.

As an attempt to clarify these points, (*R*)- and (*S*)-(*O*-^{14}Me)coclaurine, (*R*)- and (*S*)-(*N*-^{14}Me)-*N*-methylcoclaurine and the tetraoxygenated (*N*-^{14}Me) reticulines were applied to calli of *B. stolonifera* V29 which were extracted to give the five major BBIQ's after five days' growth. As expected, neither of the reticuline enantiomers was incorporated into BBIQ's, although 18% of the radioactivity of (*S*)-reticuline fed to the calli went into the protoberberine jatrorrhizine, showing that the precursor was well absorbed. The trioxygenated compounds, on the contrary, were incorporated efficiently into all five BBIQ's regardless of their configuration. Berbamunine appeared in every case to be the most radioactive product besides being the most abundant, and this dimer accounted for 10% of the radioactivity of the (*R*)-*N*-methylcoclaurine fed to the tissue (Table 4). The labelled berbamunine obtained in each feeding experiment with radioactive BIQ's was derivatized as described above to give radiometrically pure berbamunine, which was degraded by reduction with sodium in liquid ammonia [19].

As expected, (*R*)-coclaurine and (*R*)-*N*-methylcoclaurine were incorporated into the (*R*)-cleavage product [(*R*)-4′-*O*-methylarmepavine] of the dimer [82 and 70.5% radioactivity of the parent base, respectively]. Surprisingly, a considerable amount of (*R*)-coclaurine and (*R*)-*N*-methylcoclaurine were seemingly also built into the (*S*) half [(*S*)-armepavine] of the dimer [18 and 29.5% radioactivity of the parent base, respectively].

(*S*)-*N*-Methylcoclaurine and (*S*)-coclaurine were, on the other hand, not incorporated into the (*R*) half to any extent. Our ^{14}C experiments with *B. stolonifera* cell cultures show that although the utilization of the chiral trioxygenated BIQ's as building blocks in the biosynthesis of the BBIQ's is stereoselective, it seems, according to radiochemical results, to be by no means stereospecific.

In order to substantiate the ^{14}C results and to investigate the true stereochemical configuration of the dimers more closely, we applied ^{13}C labelled enantiomeric coclaurines, labelled at the chiral centre, to suspension cultures of *Berberis stolonifera* V29 cells. ^{13}C NMR spectra of the isolated dimers were recorded after work-up. The ^{13}C NMR chemical shifts of several alkaloids related to berbamine recorded at 25.2 MHz, have been assigned [20].

(1-^{13}C) (*R*)-Coclaurine was converted into berbamunine (26 ^{13}C atom % excess), the crude alkaloid showing three enriched resonances at 65.91, 65.88 and 65.83 ppm (Fig. 1.). Successive crystallizations in methanol led to products which, after ^{13}C NMR analysis, could be shown to be increasingly pure berbamunine with an isotopic excess indicative of stereospecific incorporation of the precursor. The mother liquors of the first crystallization were concentrated to afford another crop of crystals. The ^{13}C NMR spectrum of the product showed two dominant peaks of almost equal intensity at 65.86 and 65.81 ppm, suggestive of a BBIQ derived from two (*R*)-coclaurine units (**2c**). A sample of the (*R*,*R*) diastereoisomer of berbamunine, guattegaumerine, was found to be identical with the material from the berbamunine mother liquors.

The C-1 resonances of the enriched diastereomeric dimers could be effectively shifted apart after derivatization with MPTA (α-methoxy-α-trifluoromethylphenylacetic acid) [21]. TLC of the MPTA derivatives permitted separation of the diastereomeric dimers. The enantiomeric purity of the thus separated chiral BBIQ alkaloids was again confirmed by ^{13}C NMR spectroscopy (Fig. 2). Further stereochemical investigations with doubly labelled chiral coclaurines (1-^{13}C, 1-^{2}H) showed,

Table 4. Distribution of radioactivity in the bisbenzylisoquinoline alkaloids of *B. stolonifera* V29 callus cultures after feeding with ^{14}C-labelled 1-benzyl-1,2,3,4-tetrahydroisoquinolines (2.5 μCi, 35 nmol)

Precursor	Alkaloid	Amount isolated (μmol)	Rel. incorporation (%)*	Sp. act. (dpm/μmol)
(*R*)-Coclaurine†				
	2-Norberbamupine	1.82	5.1	24×10^3
	Berbamunine	2.63	8.3	27×10^3
	Aromoline	0.48	0.59	11×10^3
	Berbamine	0.39	0.44	9.9×10^3
	Isotetrandrine	0.20	0.07	3.2×10^3
(*S*)-Coclaurine†				
	2-Norberbamunine	1.5	1.47	15×10^3
	Berbamunine	1.8	6.16	53×10^3
	Aromoline	0.47	1.29	43×10^3
	Berbamine	0.51	1.67	50×10^3
	Isotetrandrine	0.10	0.19	3×10^3
(*R*)-*N*-Methylcoclaurine‡				
	2-Norberbamunine	1.1	4.51	80×10^3
	Berbamunine	3.0	29.1	190×10^3
	Aromoline	0.82	1.45	34×10^3
	Berbamine	0.84	1.21	28×10^3
	Isotetrandrine	0.36	0.17	9.4×10^3
(*S*)-*N*-Methylcoclaurine‡				
	2-Norberbamunine	4.5	0.40	2×10^3
	Berbamunine	6.7	4.37	15×10^3
	Aromoline	1.8	0.41	5.5×10^3
	Berbamine	2.2	0.68	7.2×10^3
	Isotetrandrine	0.43	0.08	3.9×10^3

* See Table 1, note *.
†(*O*-^{14}Me)Coclaurine.
‡(*N*-^{14}Me)*N*-Methylcoclaurine.

after ^{13}C NMR analysis, absolutely no loss of deuterium during incorporation into the dimers, indicating that the chiral centre remains intact during biosynthesis. These experiments with doubly labelled stable isotopes are again supportive of stereospecific incorporation of both (*R*)- and (*S*)-coclaurine enantiomers into the (*R*) and (*S*) half of the BBIQ alkaloids of *Berberis stolonifera* cell cultures.

Feeding experiments with (*S*)-*N*-^{13}Me-coclaurine showed that this isomer is incorporated exclusively into the (*S*) half of berbamunine. No indication was found of incorporation into other diastereoisomers such as (*S*,*R*)-magnoline or the enantiomer of guattegaumerine (*S*,*S*).

DISCUSSION

The fact that the specific activity of berbamunine was always greater than those of aromoline, berbamine and isotetrandrine is in accord with the suggestion that 'bases of the magnoline type and its stereoisomers are formed first in the plant' and that one of these (berbamunine) could lead to the berbamine-oxyacanthine group of alkaloids [3]. Berbamunine could thus undergo an intramolecular phenolic oxidative coupling either between C-7-OH and C-8′ to give aromoline (**5a**) or between C-7′-OH and C-8 to give obamegine (**6a**). Successive *O*-methylations of aromoline could provide oxyacanthine (**5b**) and obaberine (**5c**). Obamegine could similarly afford berbamine (**6b**) and isotetrandrine (**6c**) in that order, a hypothesis which is also borne out by the decreasing specific activities of the latter pair of alkaloids in our experiments.

2-Norberbamunine, which has recently been found as a new BBIQ in *Berberis stolonifera* cell cultures [16], could in theory be either a precursor of berbamunine or a demethylated metabolite of this compound, or both could be formed in parallel by intermolecular coupling of (*S*)-*N*-methylcoclaurine on one hand and either (*R*)-coclaurine or (*R*)-*N*-methylcoclaurine on the other. In our experiments (Table 4) the specific activities of both substances were found to be quite similar, although the percentage of incorporation of precursors into the norbase was never higher than that corresponding to its *N*,*N*′-dimethylated counterpart. This observation rules out the possibility that the *N*-methylation of norberbamunine to berbamunine is an important pathway.

Although we have not yet attempted to measure the relative sizes of the metabolic pools of (*R*)- and (*S*)-coclaurine and the (*R*)- and (*S*)-*N*-methylcoclaurines, which could determine the efficiency with which these substances are incorporated into their metabolites, it seems possible that the significantly smaller incorporation of the (*S*) enantiomers into BBIQ's is due in part to their key position on the routes leading to dimeric alkaloids and also to reticuline, as we have shown quite recently [13]. As can be seen in Table 4, (*R*)-*N*-methylcoclaurine was incorporated very well into the BBIQ's, and (*R*)-coclaurine was much less so. The (*S*)

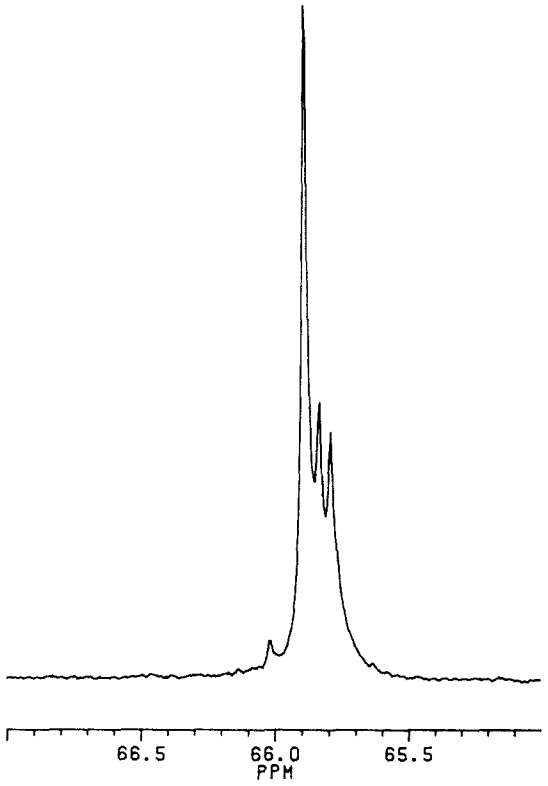


Fig. 1. Partial ^{13}C NMR spectrum of the diastereomeric dimers (berbamunine and guattegaumerine) illustrating the enriched C-1/C-1′ resonances after a (1-^{13}C) (*R*)-coclaurine feeding.

enantiomers of these monomeric BIQ's, on the contrary, appeared to be relatively poor precursors, with the *N*-unmethylated compound going in somewhat better, although *prima facie* they would be expected to go into the (*S*) half of the dimers just as well as the (*R*) precursors go into the (*R*) half (Scheme 2).

However, *B. stolonifera* cell cultures accumulate very large amounts of the reticuline metabolite jatrorrhizine [16], and this implies a considerable drain on the (*S*)-coclaurine pool. A large proportion of the latter alkaloid is converted into (*S*)-reticuline and should thus be unavailable for dimerization; it is therefore reasonable that (*S*)-coclaurine should label the BBIQ's less extensively than its enantiomer, which is not metabolized to the protoberberines. Feeding experiments have now shown that labelled (*S*)-*N*-methylcoclaurine is incorporated into the crude protoberberine alkaloid fraction even more efficiently (23.4%) than (*S*)-coclaurine (5.9%), signifying that the latter alkaloid may be *N*-methylated before hydroxylation at C-3′ on the route leading to (*S*)-reticuline. This observation may also help to explain the relatively small incorporation of (*S*)-*N*-methylcoclaurine into BBIQ's as compared with its (*R*) isomer. It should be remembered here that the more distant precursor tyramine also labels the (*R*) half of berbamunine rather more efficiently than the (*S*) half. This can also be explained by the shunting of a considerable part of the tyramine metabolites (*S*)-coclaurine and *N*-methylcoclaurine onto the (*S*)-reticuline track.

Our results with the enantiomeric (1-^{13}C)-coclaurines substantiated the usual assumption that the incorporation of these substances into the (*R*) and (*S*) halves of BBIQ's is stereospecific. The ^{13}C NMR spectra of the purified products showed that neither of the coclaurine enantiomers is converted into the other in *Berberis*, in contrast to the well-known origin of (*R*)-reticuline and the corresponding morphine alkaloids in *Papaver*, which arise from (*S*)-reticuline through 1,2-dehydroreticuline [22, 23]. They also led to the unexpected finding that the (*R,R*) diastereoisomer of berbamunine, the alkaloid guattegaumerine, is also formed in quite considerable amounts in *B. stolonifera* suspension cultures. Until now it had seemed that, with the exception of the rare C-10-

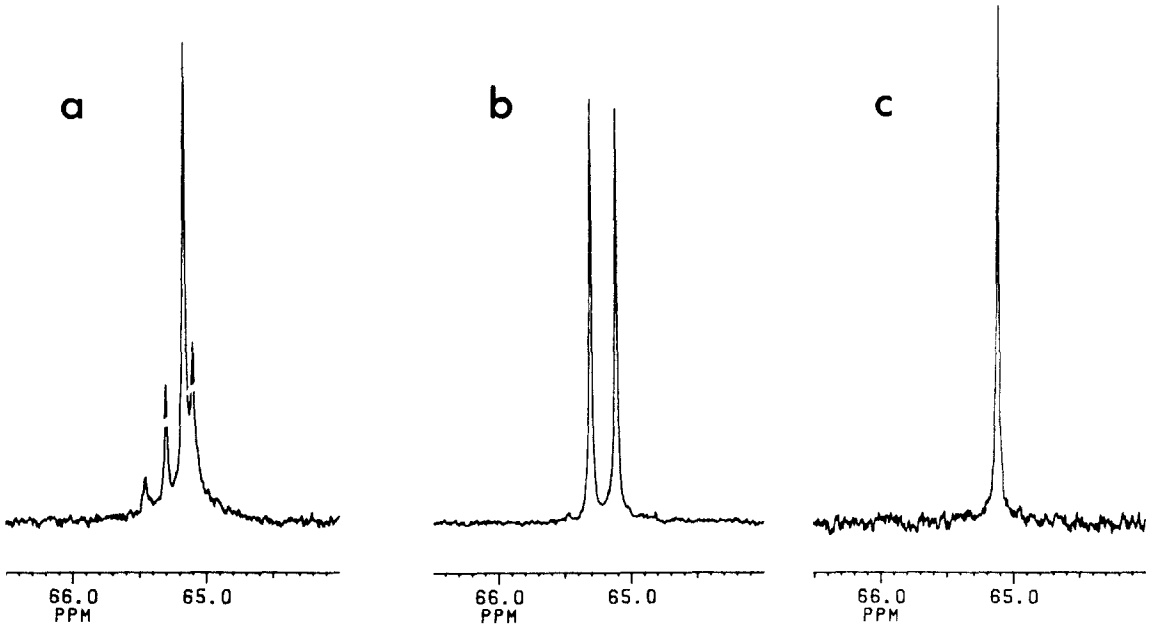
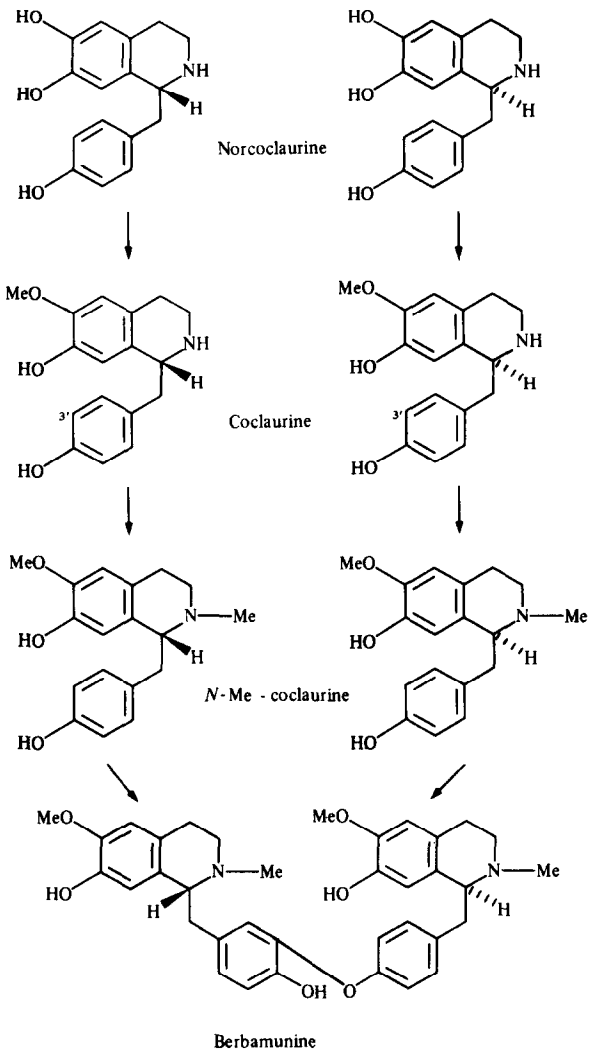


Fig. 2. Partial ^{13}C NMR spectra illustrating the enriched C-1/C-1′ resonances of (a) (*R,R*) and (*R,S*)-unresolved MPTA-derivatives; (b) (*R,R*)-MPTA derivative, 65.30 and 65.11 ppm; (c) (*R,S*)-MPTA derivative, 65.13 ppm.



Scheme 2. Biosynthetic pathway leading from (*R*)- and (*S*)-norcoclaurine to berbamunine, the chief dimer in *Berberis stolonifera* cell suspension cultures.

oxygenated (*S,R*) dimers of the osornine series [24], all the BBIQ's of *Berberis* shared the (*R,S*) absolute configuration. The isolation of guattegaumerine from *B. stolonifera* cell cultures shows that (*R,R*) dimers may also be present and should be envisioned as likely contaminants of their generally more abundant diastereoisomers. It also signals some of the risks involved in biogenetic and chemosystematic speculations based on the stereochemistry of BBIQ's found in different taxa.

EXPERIMENTAL

Plant material. Berberis stolonifera cell line V29, cultivated in this laboratory from a seedling more than 10 years ago, was propagated in Petri dishes on Linsmaier–Skoog (LS) medium [25] made solid with 1% agar and maintained in the dark at 20° and 42% relative humidity, subculturing every 3 weeks. The suspended cells were grown in LS medium in 100 ml flasks containing 25 ml medium on a gyratory shaker (100 rpm) at 23° under constant diffuse light, subculturing every 8 days using an inoculum of about 10% of the volume of the culture medium. Suspension cultures were harvested by suction filtration through nylon mesh. The dry wt of callus and suspension cultures was determined by drying aliquots at 56° for at least 2 days.

Preparation of labelled precursors. (*S*)- and (*R*)-6-*O*-^{14}Me-coclaurine were synthesized from the corresponding enantiomerically pure *nor*-compounds (2 μmol) by incubating 50 μCi (700 nmol) ^{14}Me-SAM (Amersham), ascorbate (1 mmol) KPO_4^{2-} buffer pH 7.5 (1 mmol) and 15 pkat *S*-adenosyl-L-methionine: (*R*),(*S*)-norlaudanosoline-6-*O*-methyltransferase [12] or catechol-*O*-methyltransferase (Sigma). Total volume was 5 ml. Incubation was for 4 hr at 37°.

The incubation mixture was made alkaline (pH 9.5) and extracted exhaustively with EtOAc. The organic phase was concd to dryness and the residue purified by TLC using CH_2Cl_2–MeOH–aq. NH_3 (90:9:1) (DMA-10: coclaurine R_f 0.25, norarmepavine R_f 0.45, norcoclaurine R_f 0.05, SAM R_f 0.00). The coclaurine zone was eluted and rechromatographed if necessary. Typical yields of the radiochemically pure product varied between 60 and 65% with respect to labelled SAM.

(*S*)- and (*R*)-*N*-^{14}Me-coclaurine were synthesized from the corresponding enantiomerically pure coclaurines (1 μmol) by incubating 9 μCi (125 nmol) ^{14}Me-SAM (Amersham), ascorbate (0.5 mmol) KPO_4^{2-} buffer pH 7.5 (0.5 mmol) and 650 pkat *S*-adenosyl-L-methionine: norreticuline *N*-methyltransferase from *Berberis stolonifera* [11]. Total volume was 3 ml. Incubation was for 3 hr at 30°. The incubation mixture was worked-up as above. The *N*-methylcoclaurines were chromatographed in the above solvent system (*N*-methylcoclaurine R_f 0.30). Typical yields of radiochemically pure (*R*)- and (*S*)-*N*-^{14}Me-coclaurines were 60–70% with respect to labelled SAM.

The enantiomeric purity of the coclaurines was checked by incubation with (*S*)-tetrahydroprotoberberine oxidase [26]. The (*S*) isomer was converted quantitatively to dehydrococlaurine, while the (*R*) compound was not affected at all, proving that both precursors were stereochemically pure.

Synthesis and resolution of (1-^{13}C)*-labelled* O,O'*-dibenzylcoclaurine.* (1-^{13}C) (*R,S*)-*O,O'*-Dibenzylcoclaurine was prepared by standard procedures [27]. The base of (1-^{13}C) (*R,S*)-*O,O'*-dibenzylcoclaurine (1g; 2.1 mmol) was derivatized quantitatively with (*S*)-(−)-(α)-methoxybenzylisocyanate (0.5 ml; 5 mmol) [28]. The reaction mixture was left stirring for 2 hr at room temp. The resulting ureas were separated quantitatively on a Büchi MPLC column (silica gel G60, 650 g, particle size 0.04–0.063 mm), eluting with *n*-hexane–EtOAc (1:1). The optically pure diastereoisomers (430 mg putative (*R*)-derivative, crystallized from C_6H_6–*n*-hexane as needles; mp 63–64°; 420 mg putative (*S*)-derivative, crystallized from EtOAc–*n*-hexane as needles, mp 125°) were separately fragmented with NaOBu (2 M in *n*-BuOH), refluxing for 1 hr. The enantiomers of *O,O'*-dibenzylcoclaurine hydrochloride thus obtained were hydrogenated in EtOH with 10% Pd-C catalyst. The chiral coclaurines crystallized from EtOH as needles [(*R*)-coclaurine: 165 mg, mp 246°, $[\alpha]_D$ +16.8° (MeOH; *c* 0.25). (*S*)-coclaurine: 149 mg, mp 246–247°, $[\alpha]_D$ −16.5° (MeOH; *c* 0.25)]. The optical rotations and melting points corresponding well with those in the literature [29]. The enantiomeric purity of the coclaurines was further checked by derivatization of the bases with the acid chloride of MPTA and subsequent analysis by ^{13}C NMR (solvent $CDCl_3$). The C-1 chemical shift differences of the diastereomeric MPTA derivatives are *ca* 2 ppm. The (*R*)-enantiomer reveals, as expected, only one C-1 signal at 52.61 ppm, with no resonance visible in the 54.65 ppm region, which could have corresponded to the (*S*)-enantiomer. The optical purity of the (*S*)-derivative was determined analogously. The extreme sensitivity of this analytical method enabled us to assign an enantiomeric purity of

at least 99% and probably more, to our thus purified chiral coclaurines.

Feeding procedures. Labelled (7-^{14}C)tyramine (50 μl, 2.5 μCi, 45 nmol) or (U-^{14}C)tyrosine (50 μl, 2.5 μCi, 5.0 nmol) was applied to 6-day-old calli which were then allowed to grow for another 5 days under identical conditions. The same amounts of these radioactive precursors were added to freshly subcultured cell suspensions which were grown for 4 days as described above.

The following labelled BIQ's were applied to calli under the same conditions as tyramine and tyrosine: (*R*)-(*O*-^{14}Me)coclaurine (2.5 μCi, 35 nmol), (*S*)-(*O*^{14}Me)coclaurine (2.5 μCi, 35 nmol), (*R*)-(*N*-^{14}Me)-*N*-methylcoclaurine (2.5 μCi, 35 nmol), (*S*)-(*N*^{14}Me)-*N*-methylcoclaurine, (*R*)-(*N*^{14}Me-)reticuline (1.24 μCi, 20 nmol) and (*S*)-(*N*^{14}Me)reticuline (0.20 μCi, 3.2 nmol). Labelled ^{13}C precursors (0.5 mmol/l medium) were applied to 2-day-old suspension cultured V29 cells. The cells were left to grow another 5 days under identical conditions before harvesting.

Extraction. Plant cells were suspended in MeOH (10 ml/g fr. wt), homogenized with an Ultraturrax grinder (1 min, room temp.), filtered through a fritted glass funnel, washed, resuspended in the same volume of fresh MeOH, and filtered and washed again after standing for 20 min at room temp. The combined filtrates and washings were concd to dryness under red. pres. at less than 40°, and the residues were taken up in boiling MeOH (2 × 10 ml). The MeOH solutions were concd as before, and the residues dissolved in 10 ml MeOH. An aliquot of each extract (100 μl) was mixed with Rotiszint 22 scintillation cocktail (5 ml) and ^{14}C disintegrations were counted to determine the overall incorporation.

Alkaloid isolation and quantification. Each crude extract was concentrated to dryness, dissolved in a small vol. of MeOH, spotted on 2 silica gel F_{254} precoated plastic foils (20 × 20 cm, 0.25 mm thickness), and the chromatograms were developed in an NH_3-saturated chamber using CH_2Cl_2–MeOH-aq.NH_3 (90:9:1). Alkaloid bands were located under a UV lamp and by spraying the edges of the chromatograms with iodoplatinate reagent, cut out and eluted with MeOH (2 × 10 ml). The solvent was removed and the residues were redissolved in 10 ml MeOH, and the molar concentration of BBIQ was estimated by UV spectrometry at 283 nm assuming $\varepsilon = 5000$. Radioactivity was determined as described for the crude extracts and expressed as: relative incorporation (%) = 100 × (radioactivity of the alkaloid)/radioactivity of the crude extract.

Purification to constant specific activity and degradation of the alkaloids. The larger alkaloid eluates (berbamunine and berbamine) were diluted with *ca* 5 mg of the respective unlabelled alkaloid, spotted on one silica gel foil, run with CH_2Cl_2–MeOH–aq.NH_3 (90:9:1) and eluted as described above, and the specific activities were determined. Each purified alkaloid sample was acetylated (Ac_2O–Et_3N–4-dimethylaminopyridine) and the products were purified as before and their specific activities determined. The acetyl derivatives were deacetylated and *O*-methylated in one step (CH_2N_2) and the methyl ethers were purified in the same way, determining their specific activities. The *O*,*O*′,*O*″-trimethylberbamunine (from berbamunine) and isotetrandrine (from berbamine) were oxidized with cerium (IV) ammonium nitrate and the isoquinoline and benzylic products worked up according to ref. [18] to determine their specific activities. The methylation products of berbamunine and berbamine were also cleaved with Na–liq. NH_3 [19], the major reaction products separated by prep. TLC, identified by comparison with synthetic standards, and their specific activities determined.

Derivatization with MPTA. The berbamunine base (45 mg, 0.067 mmol), extracted from the (1-^{13}C) (*R*)-coclaurine feeding to *Berberis* suspension cells, was dissolved in $CHCl_3$ and treated with MPTA Cl (50 mg, 0.2 mmol). The reaction mixture was left stirring overnight at room temp. The resulting MPTA derivatives were purified by prep. TLC on silica gel G60 precoated plates with solvent: H_2O–*n*-BuOH–acetic acid (5:4:1) yielding the enantiomerically pure (*R*,R) diastereoisomer ($R_f = 0.30$, 10 mg) and the pure (*R*,*S*)diastereoisomer ($R_f = 0.24$, 22 mg).

Determination of isotopic excess of ^{13}C *and recording of* ^{13}C *spectra.* ^{13}C enrichment was calculated from mass spectral data, (CI, EI-mode). All ^{13}C NMR spectra: 90 MHz, with solvent CD_3OD, unless otherwise specified. Mps: uncorr.

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