*Phytochemistry*, Vol. 26, No. 4, pp. 1005–1008, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

# BISBENZYLISOQUINOLINE ALKALOIDS IN *BERBERIS* CELL CULTURES

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(*Received* 8 *July* 1986)

**Key Word Index**—*Berberis* spp.; *B. stolonifera*; Berberidaceae; cell cultures; bisbenzylisoquinolines; 2-norberbamunine; alkaloid accumulation kinetics.

**Abstract**—Callus cultures of 34 *Berberis* cell lines representing 33 species were screened for alkaloids using TLC and HPLC. Jatrorrhizine was always found to be a major constituent; two *B. stolonifera* lines were the richest in bisbenzylisoquinolines. The new alkaloid 2-norberbamunine was isolated from cultures of *B. stolonifera* V29 together with berbamunine, aromoline, berbamine, isotetrandrine and jatrorrhizine. The time course of bisbenzylisoquinoline accumulation was studied in *B. stolonifera* V29 suspension cultures and it was found that the concentrations of these alkaloids rose throughout the cell growth phase.

## INTRODUCTION

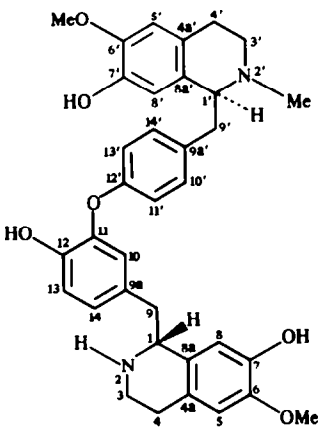
The bisbenzylisoquinolines constitute one of the largest groups of isoquinoline alkaloids, and their presence is a frequent characteristic of plants belonging to the more primitive Angiosperms. A comprehensive biogenetic hypothesis explaining the formation and structures of these compounds was formulated by Barton and Cohen as far back as 1957 [1], but experimental work on the biosynthesis of bisbenzylisoquinoline alkaloids is scarce. Incorporation studies using whole plants of *Stephania japonica* [2] or cut branches of *Cocculus laurifolius* [3–5], both of the Menispermaceae, have shown that the enantiomeric coclaurines and their *N*-methyl derivatives act as precursors of some of these dimeric alkaloids and that the stereochemistry of the monomers is conserved during their dimerization. The precise sequence of biosynthetic events, however, can be established only by work at the enzymatic level, as has been shown for the route leading from dopamine and 3,4-dihydroxyphenylacetaldehyde to berberine [6]. Nothing is yet known about the enzymology of bisbenzylisoquinoline formation, and a necessary first step in this direction is the identification of appropriate enzyme sources.

Suspension cultures of plant cells are ideally suited for the isolation of the enzymes involved in alkaloid biosynthesis [6]. As the literature records only one instance of the identification and quantification of bisbenzylisoquinolines from cultured cells (of *S. cepharantha*) [7], we decided to analyse a broader range of plant cell cultures for this important group of alkaloids. In this paper we report our results with *Berberis* species.

## RESULTS AND DISCUSSION

The genus *Berberis* is well known as a source of bisbenzylisoquinolines and their oxidized proaporphine-benzylisoquinoline, aporphine-benzylisoquinoline and seco-derivatives [8, 9]. As a sizeable collection of *Berberis* calli is kept growing in our laboratory, this genus appeared to be a good candidate for initial screening. TLC examination of a number of *Berberis* callus extracts, after partial removal of the generally abundant protoberberine alkaloids together with the neutral and acid constituents, indicated that several bases behaving chromatographically like bisbenzylisoquinolines were usually present. The highest concentrations of these substances were observed in two *B. stolonifera* cell lines designated as V29 and V30, which were therefore selected for more detailed analysis.

Extraction and fractionation of 2.4 kg (fresh weight) of suspension-cultured cells of *B. stolonifera* V29 afforded jatrorrhizine as the major alkaloidal constituent, and five biscoclaurines: isotetrandrine, berbamine, aromoline, berbamunine and the previously undescribed 2-norberbamunine (**1**). The identification of the known compounds was based on their mass spectra, $^1$H NMR spectra (at 200 MHz) and CD spectra. The EI mass spectrum of 2-norberbamunine showed an extremely faint molecular




**1**

ion peak at *m/z* 582 (composition by high resolution EIMS $C_{35}H_{38}N_2O_6$), a base peak at *m/z* 192 ($C_{11}H_{14}NO_2$ by high resolution EIMS) and a less intense (29%) peak at *m/z* 178 ($C_{10}H_{12}NO_2$ by high resolution EIMS) suggestive of one of the two possible norberbamunines or a stereoisomer of one of these structures. The 200 MHz $^1$H NMR spectrum was almost identical to that of berbamunine, but with a single *N*-methyl resonance at $\delta$2.46. Its CD spectrum in methanol showed clearly that the stereochemistry of the new alkaloid is the same as that of berbamunine.

To clarify the position of the single *N*-methyl group of the norberbamunine from *B. stolonifera*, $^1$H–$^1$H homonuclear and $^1$H–$^{13}$C heteronuclear COSY experiments were carried out, allowing unequivocal assignment of all the signals due to carbon atoms bonded to hydrogen. The amount of pure material was insufficient for the observation of long-range $^1$H–$^{13}$C couplings which would have permitted the quaternary carbon resonances to be assigned unambiguously and would also have established connectivities in a more direct fashion. $^{13}$C NMR spectra were recorded using the DEPT sequence for better identification of the carbon atoms attached to hydrogen. Our assignments, based in part on previous studies of related compounds [10–13], are summarized in Table 1.

The $^1$H NMR spectra show that one of the benzylisoquinoline halves of the norberbamunine molecule is in a folded conformation in which either H-8 or H-8′, resonating at $\delta$6.20, is shielded by ring C or ring C′, respectively. That H-8′ and ring C′ are the ones involved in this interaction is shown by the reciprocal shielding by ring A′ of the upfield ($\delta$6.73, H-11′/13′) component of the $A_2B_2$ pair of doublets. This folding is a well-known characteristic of *N*-substituted 1-benzyl-1,2,3,4-tetrahydroisoquinolines, and in the present case it strongly suggests that N-2′ is the methylated one. This interpretation was substantiated in a most striking way by the strong correlation between the *N*-methyl hydrogen atoms and H-10′/14′ and H-11′/13′ seen in the homonuclear two-dimensional NMR spectrum. The intensity of this effect, and also the shielding of the *N*-methyl group, can be attributed to a significant proportion of molecules having this substituent in a pseudo-axial orientation, recognizable by the clear $\gamma$-gauche effect on the chemical shift of C-4′ (25.23 ppm) as compared to that of C-4 (28.81 ppm).

The $^{13}$C NMR spectrum provided additional data of conformational interest. As the C-1 and C-3 resonances are shifted upfield to $\delta$56.21 and 40.80, respectively, the benzyl group at C-1 must be pseudo-axial. The chemical shifts of C-1′ and C-3′, on the other hand (63.92 and 43.36 ppm, respectively), show that the benzyl group neighbouring the pseudo-axial *N*-methyl group is pseudo-equatorial.

Once the major alkaloids of *B. stolonifera* V29 suspension cultured cells had been identified, we were able to attempt their quantification in our *Berberis* callus collection as a means of selecting other cell lines of potential utility for our biosynthetic studies. A reversed-phase HPLC gradient was developed which resolved the five dimeric alkaloids isolated from *B. stolonifera* as well as some other *Berberis* biscoclaurines available to us, and at the same time allowed jatrorrhizine, palmatine, berberine and the putative precursors coclaurine, *N*-methylcoclaurine and reticuline to be determined quantitatively. The results of the analyses of 33 crude methanolic extracts of different *Berberis* callus cultures aged between 18 and 21 days are shown in Table 2. It can be seen that both *B. stolonifera* cell lines contain the highest concentrations of bisbenzylisoquinolines and jatrorrhizine.

Table 1. $^1$H NMR (400 MHz) and $^{13}$C NMR (100.6 MHz) chemical shifts (ppm from TMS), $^1$H signal multiplicities and $^1$H–$^1$H coupling constants (Hz) for 2-norberbamunine (1) in $CDCl_3$–DMSO-$d_6$

| | $\delta^1$H | $\delta^{13}$C | | $\delta^1$H | $\delta^{13}$C |
|---|---|---|---|---|---|
| H/C-1 | 3.94 *m* | 56.21 | H/C-1′ | 3.47 *m* | 63.92 |
| $H_e$/C-3 | 3.13 *m* | 40.80 | $H_e$/C-3′ | 2.98 *m* | 47.08 |
| $H_a$/C-3 | 2.83 *m* | | $H_a$/C-3′ | 2.57 *m* | |
| $H_e$/C-4 | 2.61 *m* | 28.81 | $H_e$/C-4′ | 2.43 *m* | 25.23 |
| $H_a$/C-4 | 2.66 *m* | | $H_a$/C-4′ | 2.39 *m* | |
| C-4a | | 124.64* | C-4a′ | | 125.40* |
| H/C-5 | 6.37 *s* | 110.58 | H/C-5′ | 6.47 *s* | 111.28 |
| C-6 | | 145.18† | C-6′ | | 145.41† |
| C-7 | | 142.26‡ | C-7′ | | 143.36‡ |
| H/C-8 | 6.66 *s* | 112.40 | H/C-8′ | 6.20 *s* | 113.81 |
| C-8a | | 129.27§ | C-8a′ | | 130.39§ |
| $H_\alpha$/C-9 | 2.73 *m* | 41.16 | $H_\alpha$/C-9′ | 2.79‖ *m* | 39.47 |
| $H_\beta$/C-9 | 3.03 *m* | | $H_\beta$/C-9′ | 2.89‖ *m* | |
| C-9a | | 130.88¶ | C-9a′ | | 132.30¶ |
| H/C-10 | 6.49 *d* (2.1) | 121.39 | H/C-10′ | 7.03 *d* (8.5) | 129.64 |
| C-11 | | 146.17 | H/C-11′ | 6.73 *d* (8.5) | 116.66 |
| C-12 | | 143.74 | C-12′ | | 155.84 |
| H/C-13 | 6.75 *d* (8.5) | 116.08 | H/C-13′ | 6.73 *d* (8.5) | 116.66 |
| H/C-14 | 6.68 *dd* (8.5; 2.1) | 125.31 | H/C-14′ | 7.03 *d* (8.5) | 129.64 |
| | | | *N*-2′-Me | 2.34 *s* | 42.26 |
| *O*-6-Me | 3.70 *s* | 55.13 | *O*-6′-Me | 3.74 *s* | 55.26 |

Assignments with identical superscripts are interchangeable.

Table 2. Alkaloid content of calli of different *Berberis* cell lines (% dry wt)

| | Aromoline | 2-Norberbamunine (1) | Berbamunine | Putative oxyacanthine | Berbamine | Isotetraandrine | Jatrorrhizine | Palmatine | Berberine |
|---|---|---|---|---|---|---|---|---|---|
| *B. aggregata* | | | | | | 0.078 | 0.18 | 0.011 | 0.030 |
| *B. amurensis* | | | | | | | 1.3 | | 0.16 |
| *B. angulosa* | 0.22 | | 0.60 | | 0.80 | | 0.32 | | |
| *B. aristata** | | | | | | | | | |
| *B. arrido-callida* | | | | | | | 0.15 | | 0.013 |
| *B. beaniana* | | 0.064 | 0.16 | 0.15 | 0.22 | 0.044 | 0.15 | 0.0034 | 0.0032 |
| *B. canadensis* | 0.017 | | 0.036 | 0.056 | 0.022 | 0.011 | 0.60 | 0.011 | |
| *B. candidula* | 0.044 | | | | | 0.060 | 0.038 | | |
| *B. cerasina* | | | | 0.24 | 0.080 | | 0.20 | 0.014 | |
| *B. circumserrata* | | | | | | | 0.0032 | | |
| *B. crataegina* | | | | | | | 0.016 | 0.019 | 0.019 |
| *B. dictyophylla* | | | 0.048 | | | | 0.064 | | 0.0044 |
| *B. gagnepainii* | | | | | | | 0.18 | | |
| *B. giraldii* | | 0.056 | 0.068 | | | 0.096 | 0.80 | 0.014 | 0.068 |
| *B. henryana* | 0.24 | | 0.28 | | 0.48 | 0.19 | 1.2 | 0.014 | |
| *B. heteropoda* | | | 0.0084 | | | | 0.56 | | |
| *B. iliensis* | | | | | | | 0.036 | | 0.0022 |
| *B. julianae* | | 0.032 | 0.048 | | 0.032 | 0.044 | 0.13 | 0.0020 | |
| *B. koetiniana* | | | 0.044 | | | 0.030 | 0.10 | 0.0036 | 0.0048 |
| *B. laxiflora* | 0.020 | | 0.10 | 0.26 | 0.14 | | 0.90 | 0.018 | 0.0010 |
| *B. mucrifolia* | | | | | | | 0.16 | | |
| *B. notabilis* | 0.0084 | | 0.10 | 0.060 | 0.024 | | 0.22 | 0.010 | 0.0002 |
| *B. nummularia* | 0.013 | | 0.031 | 0.032 | 0.026 | | 0.034 | | |
| *B. papillifera* | | 0.024 | 0.020 | | | | 0.064 | | |
| *B. paravirescens* | | | | | | | 0.080 | 0.0030 | 0.0008 |
| *B. serrata* | | | 0.026 | | | | 0.17 | | 0.0044 |
| *B. sieboldii** | | | | | | | | | |
| *B. stenophylla* | | | | | 0.0036 | | 0.13 | | |
| *B. stolonifera* V29 | 0.28 | 0.96 | 0.94 | 0.060 | 0.40 | 0.060 | 2.6 | 0.011 | 0.019 |
| *B. stolonifera* V30 | 0.26 | 1.1 | 0.66 | | 0.56 | 0.096 | 3.0 | 0.038 | |
| *B. taliensis* | 0.086 | 0.056 | 0.032 | 0.58 | | 0.12 | 1.6 | 0.052 | |
| *B. turcomanica* | 0.062 | 0.030 | 0.36 | 0.040 | 0.10 | 0.14 | 0.30 | 0.015 | 0.0032 |
| *B. vernae* | | | | | | | 0.070 | 0.022 | 0.044 |
| *B. wilsonae* | | 0.10 | 0.36 | 0.034 | | | 0.12 | | 0.030 |

*No identified alkaloids.

The same HPLC method was used to study the accumulation of alkaloids in suspension-cultured cells of *B. stolonifera* V29. The results are shown in Fig. 1, which suggest that the concentrations of all the bisbenzylisoquinolines increase steadily from the first to the ninth day of growth or shortly thereafter, following the cell growth curve quite closely. By the ninth day some cultures had blackened, and the analytical results varied considerably from one flask to another. By the eighteenth day, most of the cultures had died.

Under the applied growth conditions, the rate of bisbenzylisoquinoline alkaloid formation appeared to be fairly constant during the first 9 days, degradative processes becoming more important thereafter. It would thus seem advisable to carry out incorporation studies within the active growth phase, attempting the isolation of enzymes from suspension cells in their second week. Research is in progress to elucidate the biosynthesis of berbamunine, by far the most abundant bisbenzylisoquinoline in *B. stolonifera* cell cultures.

## EXPERIMENTAL

*Plant materials.* Callus cultures initiated from seedlings several years back were maintained on Linsmaier–Skoog medium at 23° under diffuse light, subculturing every 2 weeks. The dry wt of these cultures were determined on aliquots dried at 50° for at least 2 days. The suspension-cultured cells used for isolation and characterization of the alkaloids were grown for 10 days in Linsmaier–Skoog medium under the same temp. and light conditions as the calli, in 1 l. batches which were harvested by suction filtration through nylon mesh, frozen in liquid $N_2$, combined, and stored at −20°. The kinetic study was carried out by seeding the same culture medium (25 ml portions) with suspension cells (5 g), growing as above, and harvesting in the same way at 1, 2, 3, 6, 9, 13 and 18 days.

*Extraction.* Calli and filtered suspension-cultured cells for TLC and HPLC analyses (2–10 g) were suspended in MeOH (10 ml/g fr. wt), disrupted with an Ultraturrax grinder (1 min) at room temp. and filtered through a fritted glass funnel, washing with the same solvent. The residue was resuspended in a fresh

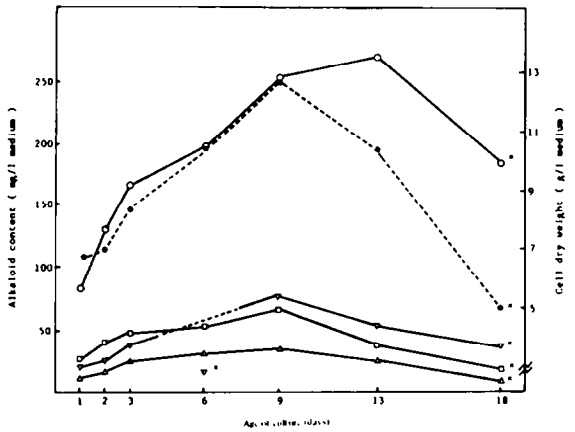


Fig. 1. Time course of alkaloid concentration and cell dry wt in suspension cultures of *B. stolonifera* V29. ○, Berbamunine; □, 2-norberbamunine (1); ▽, berbamine; △, aromoline; ●, cell dry wt. All points represent the average of two determinations, except those marked × (one determination).

portion of MeOH, allowed to stand for 15–30 min, and filtered and washed as before. The combined filtrates and washings were concentrated to dryness under red. pres. at less than 40°, and the residues were taken up in 10 ml boiling MeOH for HPLC. TLC analyses of selected cultures were performed on the crude alkaloids obtained by partitioning the above-mentioned residue between $Na_2SO_4$-saturated 5% citric acid (10 ml) and $CH_2Cl_2$ (20 ml, then twice with 10 ml), raising the pH of the aq. phase to 8.5, and extracting the bases with $CH_2Cl_2$ (3 × 10 ml). After removing the solvent, the residues were taken up in MeOH (0.1 ml/g fr. wt).

*TLC.* Each fraction was spotted on silica gel $F_{254}$ precoated plastic foil, washed previously with MeOH, developing with $CH_2Cl_2$–MeOH–aq. $NH_3$ (90:9:1) in an $NH_3$-saturated chamber. Alkaloids were visualized by UV absorption and by spraying with iodoplatinate reagent.

*HPLC.* Samples of the MeOH extracts (20 μl) were chromatographed at room temp. using a Vydac SC-201 RP (Macherey-Nagel) guard column (30 × 4.6 mm) and a 5 μm Nucleosil $SC_{18}$ analytical column (250 × 4.6 mm). Analyses were carried out eluting with a 0.01% (v/v) $H_3PO_4$–MeCN gradient going from 10% to 35% MeCN in 35 min, and then to 80% MeCN in 10 min, detecting the alkaloid peaks spectrophotometrically at 282 nm.

*Alkaloid isolation and identification. B. stolonifera* V29 cells (2.4 kg fr. wt, equivalent to approximately 0.12 kg dry wt) were suspended in 3 l. MeOH, homogenized in a blender, diluted with MeOH to a final vol. of 7.5 l., and filtered and washed with MeOH after standing overnight at room temp. The residue was resuspended in 2.5 l. MeOH, left at room temp. for 24 hr, filtered and washed. The combined filtrates and washings were concentrated under red. pres. at less than 40° to a vol. of 0.5 l., diluted with $H_2O$ to 1 l., acidified by addition of solid citric acid (50 g), and extracted with $CH_2Cl_2$ (2 × 1 l.), discarding the orange-yellow organic layers. The pH of the aq. soln was adjusted to 8.5 with solid $Na_2CO_3$ and the basic soln was extracted with $CH_2Cl_2$ (3 × 1 l.), drying and removing the solvent immediately. The crude alkaloids (3.5 g, 3% based on dry wt) were adsorbed on 11.3 g silica gel 60 (particle size 0.040–0.063 mm) and placed on a column of the same material (100 g) packed in $CH_2Cl_2$–MeOH–aq. $NH_3$ (90:9:1), eluting with the same solvent mixture. Fractions (25 ml) were collected and analysed by TLC. Fractions 6–9 (82 mg) contained mainly isotetrandrine; fractions 10–17 (400 mg) consisted mainly of berbamine; fractions 18–21 (98 mg) contained approximately equal amounts of berbamine and aromoline; in fractions 22–25 (107 mg) aromoline predominated; fractions 26–28 (120 mg) contained similar proportions of aromoline and berbamunine; fractions 29–41 (840 mg) consisted mainly of berbamunine; fractions 42–52 (1.29 g) also contained berbamunine as the principal constituent, but 2-norberbamunine was present in isolable amounts. Concentration of the later orange-coloured fractions afforded crystals of jatrorrhizine. Prep. TLC of the bisbenzylisoquinoline-containing fractions allowed purification of the bases mentioned above.

The previously known compounds were identified by MS, 200 MHz $^1$H NMR, CD and TLC comparison with reference samples.

*2-Norberbamunine* (1). Amorphous. EIMS *m/z* (rel. int.): 582.2697 $[M]^+$ (0.14), $C_{35}H_{38}N_2O_6$ calc. 582.2709; 405.1955 (7.04), $C_{25}H_{27}NO_4$ calc. 405.1970; 192.0990 (100), $C_{11}H_{14}NO_2$ calc. 192.0956; 178.0869 (29.55), $C_{10}H_{12}NO_2$ calc. 178.0869; NMR: see Table 1; CD (MeOH) $\Delta\varepsilon$ (nm) 0 (300), +1.93 (285), +0.55 (262), +6.6 (242), descending tail.

*Acknowledgements*—We are grateful to Dr. H.-M. Schumacher for the low resolution mass spectra, Dr. A. Treiber for the 200 MHz $^1$H NMR spectra, Dr. G. Eckhardt for the high resolution EI mass spectra, and Mr. E. Gessi for the CD spectra. B.K.C. thanks the Alexander von Humboldt-Stiftung for a fellowship. This work was supported by the Sonderforschungsbereich 145 of the Deutsche Forschungsgemeinschaft.

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