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NEW HYDROXYLATED BENZO[*c*]PHENANTHRIDINE ALKALOIDS FROM *ESCHSCHOLTZIA CALIFORNICA* CELL SUSPENSION CULTURES

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ABSTRACT.—From cell cultures of *Eschscholtzia californica* and their spent medium, three new benzo[*c*]phenanthridine alkaloids—namely 10-hydroxysanguinarine [**2a**], 12-hydroxychelirubine [**4a**], and 10-hydroxychelerythrine [**7a**]—and two new dihydrobenzo[*c*]phenanthridine alkaloids—10-hydroxydihydrosanguinarine [**2b**] and 12-hydroxydihydrochelirubine [**4b**]—together with the known constituents sanguinarine [**1a**], chelirubine [**3a**], macarpine [**5a**], dihydrosanguinarine [**1b**], dihydrochelirubine [**3b**], and dihydromacarpine [**5b**], were isolated and characterized. Structure elucidations were done by ^{1}H nmr, decoupling experiments, and NOESY spectra. Isolated microsomes from *E. californica*, the site of hydroxylation activity within the cells, contained the whole set **1b** to **8b** of 5,6-dihydrobenzo[*c*]phenanthridines. A scheme for the biosynthesis of macarpine [**5a**] from protopine [**9**] via dihydrosanguinarine [**1b**] is presented.

The biosynthesis of benzo[*c*]phenanthridine alkaloids from L-tyrosine presents an intriguing biochemical problem (1). Protopine [**9**] and sanguinarine [**1a**] are the generally accepted precursors for most higher substituted benzophenanthridines, and a pathway for the formation of the most highly oxidized benzophenanthridine alkaloid, macarpine [**5a**], has been postulated on the basis of precursor feeding experiments (2).

A chance observation, the infection of a callus culture of a colorless strain of *Eschscholtzia californica* by a *Penicillium* fungus resulting in a strong red zone at the contact site between both organisms, led to the discovery that this callus culture can easily be elicited to produce considerable amounts of the quaternary alkaloid macarpine as well as other benzophenanthridines (3). A different cell culture strain of *E. californica* had previously been investigated (4) for its dihydrobenzophenanthridine content. The abovementioned elicitation phenomenon was later studied by other workers using a different *E. californica* cell culture strain (5). The crucial step in the biosynthesis of benzophenanthridine alkaloids has been recently discovered (6). Protopine [**9**] is hydroxylated at the C-6 position by a microsomal stereo- and regio-specific cytochrome P-450 enzyme. 6-Hydroxyprotopine undergoes a (most likely spontaneous) ring opening, rearrangement, and dehydration yielding dihydrosanguinarine [**1b**] (6). The more highly oxidized members of the benzophenanthridine family found in *Macleaya cordata* or *E. californica* are derived either from sanguinarine (2) or from dihydrosanguinarine (6). Chelirubine [**3a**] has previously been postulated (2) as the sole intermediate between sanguinarine [**1a**] and macarpine [**5a**]. In continuation of our analysis of the elicitation phenomenon in *E. californica* cell cultures, we investigated the occurrence of benzophenanthridines in this strain and discovered all of the hitherto missing potential intermediates between dihydrosanguinarine and macarpine.

RESULTS AND DISCUSSION

In the course of isolating the known (3,4) benzo[*c*]phenanthridine constituents of *E. californica* cell cultures that were either deposited in the cells or released into the growth medium, we consistently encountered additional alkaloids which belonged to this class of compounds.

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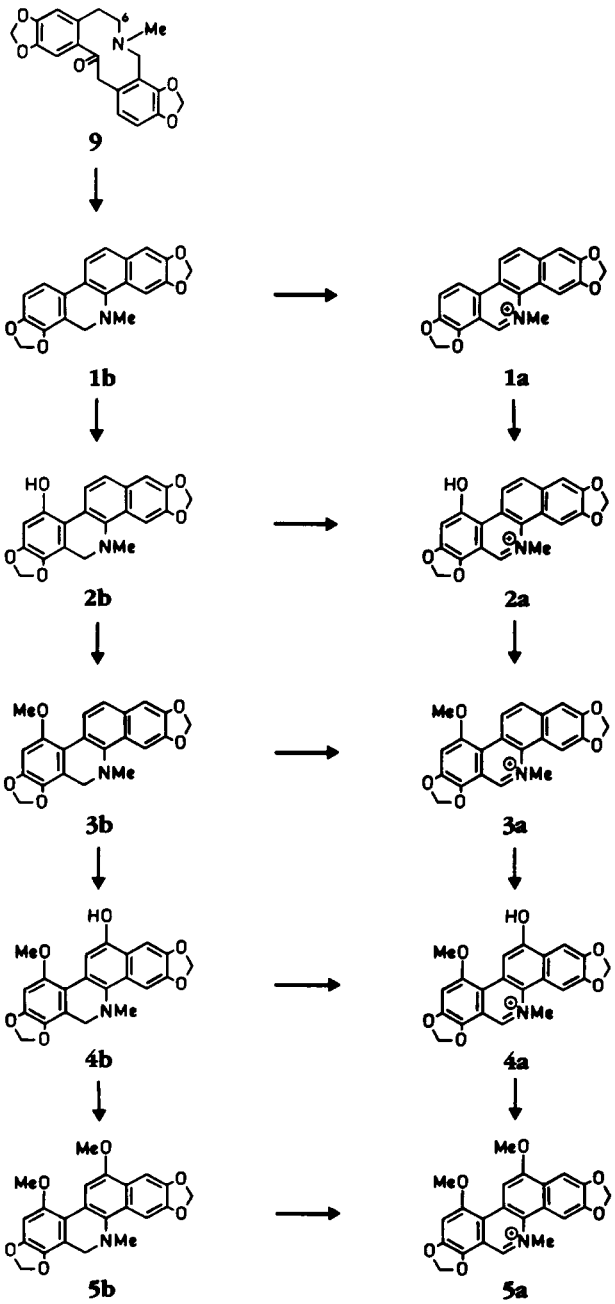
Fractionation of the cultured medium led to the isolation of three new benzo[*c*]phenanthridine alkaloids, 10-hydroxysanguinarine [**2a**], 12-hydroxychelirubine [**4a**], and 10-hydroxychelerythrine [**7a**], together with the known constituents sanguinarine [**1a**], chelirubine [**3a**], and macarpine [**5a**].

The first new alkaloid **2a** was isolated as a dark red crystalline solid. Its uv spectrum [λ max (MeOH) 229, 281, 304 sh, 343, 355, 414 sh, 508] was typical of a benzophenanthridine (7). The ^{1}H-nmr spectrum of **2a** (Table 1) revealed the presence of NMe and two methylenedioxy substituents. The aromatic region of the spectrum



SCHEME 1

TABLE 1. ^{1}H-nmr Data for the Isolated Benzo[c]phenanthridine Alkaloids.[a]

Proton	Compound					
	1a[b]	**2a**	**3a**	**4a**	**5a**	**7a**
H-1	7.78 s	7.70 s	7.73 s	7.71 s	7.75 s	7.72 s
H-4	8.28 s	8.15 s	8.17 s	8.14 s	8.15 s	8.21 s
H-6	10.15 s	9.98 s	10.05 s	9.74 s	9.83 s	9.97 s
H-9	8.13 d (8.8)	7.60 s	7.98 s	7.88 s	7.92 s	7.68 s
H-10	8.65 d (8.8)	—	—	—	—	—
H-11	8.80 d (9.0)	9.54 d (9.2)	9.38 d (9.1)	8.93 s	8.87 s	9.58 d (9.2)
H-12	8.31 d (9.0)	8.20 d (9.2)	8.22 d (9.1)	—	—	8.23 d (9.2)
N-Me . . .	4.92 s	4.82 s	4.85 s	4.82 s	4.82 s	4.91 s
O-Me . . .	—	—	4.17 s	4.15 s	4.17 s[12-OMe]	4.04 s
	—	—	—	—	4.20 s[10-OMe]	4.05 s
OCH_2O . .	6.35 s	6.33 s	6.34 s	6.33 s	6.34 s	6.33 s
	6.61 s	6.50 s	6.56 s	6.52 s	6.54 s	—
OH	—	11.85 br s	—	11.53 br s	—	11.85 br s

[a]Recorded at 360 MHz in DMSO-d_6. Chemical shifts are given in δ (ppm) relative to TMS. Values in parentheses are coupling constants in Hz. Assignments were made with the aid of NOESY spectra.

[b]A revision of the earlier assignments (11). Our assignments are based on the NOESY spectrum of **1a**, which showed nOe enhancements between H-12 (δ 8.31) and H-1 (δ 7.78), H-11 (δ 8.80) and between H-10 (δ 8.65) and H-9 (δ 8.13), H-11 as well as nOe's between N-Me (δ 4.92) and H-4 (δ 8.28), H-6 (δ 10.15).

showed six protons, four as singlets and a pair of doublets for two ortho-coupled protons. This pattern of aromatic protons and substituents was closely similar to that of chelirubine except for the chemical shift of H-9. Another significant difference in their ^{1}H-nmr spectra was that **2a** contained no methoxyl signal but a phenolic proton (δ 11.85). A mass spectrum of the isolate showed an $[M]^+$ at *m/z* 348, which was 14 mass units lower than that of chelirubine ($[M]^+$ *m/z* 362).

As an additional structural confirmation, **2a** was reduced with $NaBH_4$ to the dihydro analogue **2b**, which was treated with CH_2N_2/Et_2O to yield the *O*-methylated product, found to be identical with an authentic sample of dihydrochelirubine [**3b**]. Thus, the new alkaloid could unequivocally be assigned to 10-hydroxysanguinarine [**2a**].

The second alkaloid **4a** was obtained as a light red crystalline solid. The ^{1}H-nmr spectrum of this compound was comparable with that of macarpine [**5a**] except for the absence of one methoxyl signal and the presence of one hydroxyl proton. These observations, together with the mass spectral data, suggested that the unknown compound represents *O*-demethylmacarpine [**4a**]. This proposal was substantiated by the reduction of **4a** and subsequent *O*-methylation of the resulting dihydro derivative **4b** to dihydromacarpine [**5b**].

The placement of the methoxyl and hydroxyl groups in **4a** was determined by comparison of the ^{1}H-nmr chemical shifts for the methoxyl signals and aromatic protons of **4b** and **5b** (Table 2). In the spectrum of **4b**, the methoxyl group resonated at the same frequency as that at C-10 in **5b**. H-1 and H-11 were affected by *O*-methylation, shifting downfield from δ 7.46 to δ 7.53 and δ 7.77 to δ 7.82, respectively, while H-9 revealed no appreciable difference. These findings reflect the fact that the phenolic function in **4b** is located at C-12 rather than at C-10. This conclusion was further supported by the NOESY spectrum of **4a**, in which nOe's were observed between the *O*-methyl group and the aromatic proton at δ 7.88 (H-9) and between the methyl signal and the

TABLE 2. ^{1}H-nmr Data for the Isolated and Related Dihydrobenzo[*c*]phenanthridine Alkaloids.[a]

Proton	Compound							
	1b[b]	**2b**	**3b**[b]	**4b**	**5b**[b]	**6b**[c]	**7b**[c]	**8b**[d]
H-1	7.11 s	7.12 s	7.10 s	7.46 s	7.53 s	7.11 s	7.11 s	7.11 s
H-4	7.67 s	7.68 s	7.69 s	7.67 s	7.66 s	7.67 s	7.68 s	7.69 s
H_2-6	4.20 s	4.09 s	4.10 s	4.08 s	4.09 s	4.29 s	4.19 s	4.22 s
H-9	6.86 d (8.1)	6.45 s	6.61 s	6.60 s	6.61 s	6.95 d (8.5)	6.47 s	6.57 s
H-10	7.30 d (8.1)	—	—	—	—	7.51 d (8.5)	—	—
H-11	7.69 d (8.6)	8.14 d (8.6)	8.30 d (8.8)	7.77 s	7.82 s	7.70 d (8.5)	8.10 d (8.6)	8.29 d (8.5)
H-12	7.48 d (8.6)	7.51 d (8.6)	7.46 d (8.8)	—	—	7.48 d (8.5)	7.50 d (8.6)	7.46 d (8.5)
N-Me	2.62 s	2.60 s	2.58 s	2.52 s	2.53 s	2.60 s	2.57 s	2.56 s
O-Me	—	—	3.87 s	3.88 s	3.88 s [10-OMe]	3.88 s [7-OMe]	3.81 s [7-OMe]	3.82 s
	—	—	—	—	4.00 s [12-OMe]	3.93 s [8-OMe]	3.89 s [8-OMe]	3.93 s
	—	—	—	—	—	—	—	3.95 s
OCH_2O	6.04 s	5.99 s	6.01 s	6.00 s	6.01 s	6.05 s	6.05 s	6.04 s
	6.05 s	6.05 s	6.04 s	6.05 s	6.04 s	—	—	—
OH	—	5.12 br s	—	n.d.[e]	—	—	n.d.[e]	—

[a]Recorded at 360 MHz in $CDCl_3$. Chemical shifts are given in δ (ppm) relative to TMS. Values in parentheses are coupling constants in Hz.

[b]Assignments according to Takao *et al.* (12).

[c]Assignments were made with the aid of NOESY spectra.

[d]Derived from **7b**; assignments according to Ishii *et al.* (13).

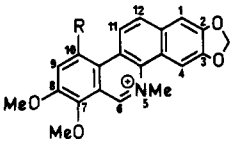
[e]n.d. = not detected.

proton resonating at δ 8.93 (H-11). Therefore, the new alkaloid **4a** was characterized as 12-hydroxychelirubine [**4a**].

The third alkaloid **7a** was isolated as an orange crystalline solid. The corresponding ^{1}H-nmr spectrum exhibits a similarity in the pattern of the aromatic protons to that of **2a**. However, in contrast to **2a**, this compound revealed only one methylenedioxy signal and two methoxyl signals, suggesting an *O*-demethyl derivative of chelilutine [**8a**], which had been already detected along with chelerythrine [**6a**] in the same cell line (3). Preparation of dihydrochelilutine [**8b**] from **7a** through $NaBH_4$ reduction and subsequent methylation substantiated this proposal. The NOESY spectrum of the dihydro derivative **7b** of **7a** showed nOe enhancements between OMe (δ 3.81) and H_2-6 (δ 4.19) and between OMe (δ 3.89) and H-9 (δ 6.47) as well as nOe interactions between NMe and H-4, H_2-6 and between H-12 and H-1, H-11; however, no nOe was observed between OMe (δ 3.89) and H-11 (δ 8.10). Although these findings led to the assumption that **7a** should be 10-*O*-demethylchelilutine, the possibility of 8-*O*-demethylchelilutine could not be excluded. The position of the hydroxy group in **7a** was finally determined by comparative studies of the ^{1}H-nmr signals of **1b**, **2b**, **3b**, dihydrochelerythrine [**6b**], and dihydro derivatives **7b** and **8b** (Table 2). When a hydroxy group was introduced at C-10 of dihydrosanguinarine, H_2-6 and H-9 were shifted upfield by 0.11 and 0.41 ppm, respectively, and H-11 was shifted downfield by 0.45 ppm. Methylation of the hydroxyl group effected a distinct downfield shift of 0.16 ppm of both H-9 and H-11 signals. Similar trends were observed with **6b**, **7b**, and **8b**. These observations could only be explained by the location of the hydroxyl substituent at C-10

in **7b**, in analogy with **2b**. Thus, the structure of this new compound was elucidated as 10-*O*-demethylchelilutine, i.e., 10-hydroxychelerythrine.

From cultured cells of *E. californica* the dihydrobenzophenanthridine alkaloids dihydrosanguinarine [**1b**], 10-hydroxydihydrosanguinarine [**2b**], dihydrochelirubine [**3b**], and dihydromacarpine [**5b**] were isolated in addition to the previously mentioned benzophenanthridine alkaloids. Of the four dihydro derivatives, 10-hydroxydihydrosanguinarine represents a novel compound. The structure was determined by comparison with the dihydro derivative of **2a**.



6a R=H
6b R=H, 5,6-dihydro
7a R=OH
7b R=OH, 5,6-dihydro
8a R=OMe
8b R=OMe, 5,6-dihydro

Based mainly on our general biosynthetic interest in the benzophenanthridines, we decided to investigate the endogenous alkaloids of a microsomal preparation of *E. californica*. Also, these fractions are known to harbor protopine-6-hydroxylase, the key enzyme in benzophenanthridine biosynthesis (6). The hplc profile (Figure 1) of the MeOH extract of the 48,000*g* pellet depicted peaks which could be attributed to 10-hydroxydihydrosanguinarine [**2b**] and 12-hydroxydihydrochelirubine [**4b**] as well as **1b**, **3b**, and **5b**. The endogenous amounts of **2b** and **4b** were 1.0 nmol/mg protein and 6.1 nmol/mg protein, respectively. The hplc peak of **7b** was also detected, accompanied by small peaks which most likely corresponded to **6b** and **8b**. A definite decrease of **4b** was observed when the extract was allowed to stand at 4° for 2 days, probably due to its instability. This may explain why we were not able to isolate 12-hydroxydihydrochelirubine from cell cultures of *E. californica*.

The co-occurrence of a variety of hydroxylated and methoxylated benzophenanthridines **1–5** in *E. californica* cell cultures now allows the formulation of a general biosynthetic pathway leading from protopine [**9**] to macarpine [**5a**] (Scheme 1). The 6-hydroxylation of protopine [**9**] leads to dihydrosanguinarine [**1b**] (6). This reaction takes place in a microsomal fraction with the cells and is catalyzed by a cytochrome P-450 enzyme. Hplc examination of this microsomal fraction led now to the discovery (Figure 1) that at least eight dihydrobenzophenathridine alkaloids (both hydroxylated and methoxylated derivatives) are present while the 5,6-dehydro derivatives **1a–5a** could not be detected. This points to the fact that dihydrosanguinarine [**1b**] is metabolized by stepwise and alternating hydroxylation and methylation reactions. We therefore postulated that macarpine, the major alkaloid of *E. californica* suspension cultures (3), originates from dihydrosanguinarine [**1b**] via 10-hydroxydihydrosanguinarine [**2b**], dihydrochelirubine [**3b**], and 12-hydroxydihydrochelirubine [**4b**] to dihydromacarpine [**5b**]. Each of these dihydrobenzophenanthridine alkaloids **1b** to **5b** can be oxidized by the cytosolic enzyme dihydrobenzophenanthridine oxidase (8) to yield the quaternary alkaloids **1a** to **5a**, which are stored within the cell or partly released into the culture medium. Elicitation of the plant cells by fungal cell wall components initiates benzophenanthridine synthesis. Benzo[*c*]phenanthridines are phytoalexins (9). The antimicrobial activity of benzophenanthridine alkaloids has also been

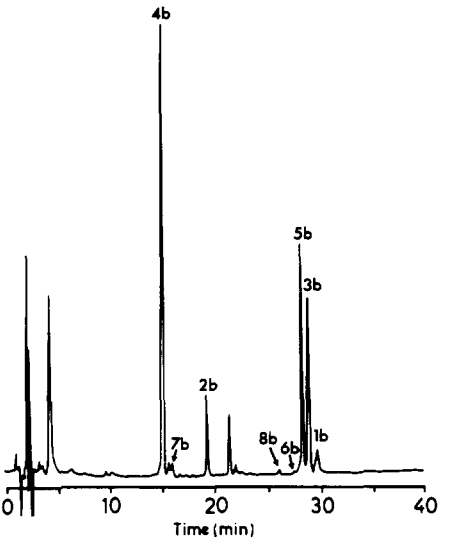


FIGURE 1. Hplc elution profile of the endogenous alkaloids in 48000*g* (microsomal) pellet from the elicited cell cultures of *Eschscholtzia californica*. Column: Nucleosil C_{18} (4.6 mm × 25 cm). Solvent systems A = H_2O-MeCN-H_3PO_4 (98:2:0.01), B = MeCN-H_2O-H_3PO_4 (90:10:0.01); linear programmed gradient from 0 to 20 min (35–75% B), from 20 to 40 min (75–100% B). Flow rate: 1.5 ml/min. Detection: 280 nm. **1b** = dihydrosanguinarine, **2b** = 10-hydroxydihydrosanguinarine, **3b** = dihydrochelirubine, **4b** = 12-hydroxydihydrochelirubine, **5b** = dihydromacarpine, **6b** = dihydrochelerythrine, **7b** = 10-hydroxydihydrochelerythrine, **8b** = dihydrochelilutine.

documented (10). The mechanism of action of these interesting alkaloids on microbial metabolism has been investigated in our laboratory and will be reported on in due course. Clearly, benzophenanthridine alkaloids are defense compounds and support plants in their struggle for life in the ecosystem.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were measured on a Kofler hot stage and are uncorrected. Uv spectra were recorded with a Kontron Uvikon 810 and ir spectra on a spectrometer Accu Lab 1 from Beckman. ^{1}H-nmr measurements were performed with a Bruker AM 360 instrument at 360 MHz. Eims (70 eV) were obtained on a Finnigan MAT 44S spectrometer and recorded up from *m/z* 100. Hplc was conducted on an instrument consisting of Waters 600 Multisolvent Delivery System, WISP Model 710B, Lambda-Max Model 481 LC spectrometer, and Waters 740 Data Module. The system employed a Nucleosil C_{18} (10 μm, 4.6 mm × 25 cm, Bischoff) column connected with a precolumn (4.6 mm × 4 cm) containing Vydac SC-201 RP (30–40 μm). Cc was conducted on Merck Si gel 60 (30–70 mesh). Analytical tlc was performed on Polygram, Sil G/UV_{254} (Macherey-Nagel) and preparative tlc on Si gel GF_{254} precoated glass plates with 0.25 or 0.5 mm thickness (Merck). The spots were visualized under uv light (254 and 366 nm).

CELL CULTURES.—A culture of *E. californica*, which was established on Linsmaier and Skoog (LS) (14) medium, was split by cellular cloning into different cell lines that varied in their content of benzo[*c*]phenanthridines and their ability to be stimulated by fungal elicitors (3). Only that strain appearing colorless in visible light was used in this study. The suspension cultures were transferred every 7th day and were cultivated at 100 rpm at 23° under 600 lux incandescent light.

PRODUCTION OF BENZO[*c*]PHENANTHRIDINE ALKALOIDS.—A 7-day-old culture (each ca. 200 ml, ca. 2 g dry wt) of *E. californica* was inoculated into eight Fernbach flasks, each containing 1000 ml LS medium. After 4 days of agitation (100 rpm) at room temperature, sterile solutions of baker's yeast elicitor (3) (60 mg in 6 ml H_2O) and $MnCl_2 \cdot 4H_2O$ (1.2 g in 6 ml H_2O) were introduced into each of the flasks. Incubation was then continued for another 3 days, after which culture medium (ca. 10 liters) and cell materials (fresh wt 845 g) were separately harvested by suction.

ISOLATION OF ALKALOIDS.—*Cell culture medium*.—The red-colored medium acidified with concentrated HCl (1 ml/liter medium) was subjected to a Servachrome XAD cc (6 × 16 cm), which was eluted with H_2O-concentrated HCl (1000:1) (1 liter); MeOH-H_2O-concentrated HCl (100:900:1) (1 liter), (200:800:1) (1 liter), (300:700:1) (1 liter), (400:600:1) (4 liters), (500:500:1) (5 liters), (600:400:1) (1 liter), (700:300:1) (1 liter), (800:200:1) (2 liters); and MeOH-concentrated HCl (1000:1) (2 liters). Fractions of 1 liter were collected and monitored by tlc [SiO_2; *n*-hexane–EtOAc (5:1 or 5:2)] after $NaBH_4$ reduction of small portions of the eluates. Those fractions, when combined on the basis of tlc characteristics, gave four major fractions as follows: M-1 (135 mg; fractions 8–10; major alkaloids **1a**, **2a**), M-2 (69 mg; fractions 11–13; **1a**, **2a**, **3a**, **4a**, **5a**, **7a**), M-3 (181 mg; fractions 14–17; **3a**, **4a**, **5a**), M-4 (289 mg; fractions 18–19; **4a**). Each of the fractions was repeatedly chromatographed on Sephadex LH-20 (5 × 52 cm or 3 × 64 cm) eluting with MeOH-H_2O-concentrated HCl (500:500:1 or 800:200:1). The isolated compounds were eluted in the following order: **1a** (1.2 mg), **3a** (9.8 mg), **5a** (19.3 mg), **7a** (3.5 mg), **4a** (116.0 mg), and **2a** (42.7 mg).

Cultured cells.—The filtered suspension-cultured cells were extracted with hot MeOH (1 liter × 4). The MeOH extracts were combined and concentrated in vacuo to an aqueous suspension (ca. 200 ml), which was filtered through a celite layer. After washing with H_2O, the insoluble material residing on the celite layer was extracted exhaustively with C_6H_6-MeOH (1:1). The extracts were pooled and evaporated to yield a gummy residue (5.00 g). A portion (0.75 g) of the aforementioned material was chromatographed on Si gel (20 g). Combined fractions eluted with C_6H_6, C_6H_6-Et_2O (99:1 to 96:4), and C_6H_6-MeOH (9:1) were concentrated in vacuo to afford residues C-1 (281 mg), C-2 (65 mg), and C-3 (229 mg), respectively. Residue C-1 was subjected to Si gel cc followed by preparative tlc [*n*-hexane–EtOAc (5:1)] to give **1b** (3.7 mg), **3b** (16.6 mg), and **5b** (23.0 mg). Residue C-2 was purified by preparative tlc [*n*-hexane–EtOAc (5:2)], yielding **2b** (3.2 mg). Residue C-3 was chromatographed on Sephadex LH-20 (5 × 52 cm) eluting with MeOH-concentrated HCl (1000:1) to give a benzophenanthridine alkaloids fraction (100.5 mg). Hplc of this fraction showed peaks corresponding to **1a**, **2a**, **3a**, **4a**, **5a**, and **7a** with relative intensities of 1.8, 15.5, 10.9, 29.7, 31.2, and 6.8% (280 nm), respectively.

10-Hydroxysanguinarine chloride [**2a**].—Dark red crystals from MeOH/dilute HCl: mp 230–231° (dec); uv (MeOH) λ max nm (log ε) 229 (4.56), 281 (4.50), 304 sh (4.00), 343 (4.26), 355 (4.28), 414 (2.82), 508 (3.10); ir (KBr) ν max cm^{-1} 3440, 1632, 1613, 1600, 1500, 1470, 1347, 1322, 1255, 1209, 1035; eims *m/z* (rel. int. %) $[M]^+$ 348 (13), 347 (8), 333 (88), 304 (20), 275 (13), 247 (52), 189 (38), 167 (63), 166 (59), 138 (71), 137 (66), 123 (100), 109 (88); ^1H nmr see Table 1.

12-Hydroxychelirubine chloride [**4a**].—Light red crystals from MeOH/concentrated HCl. This compound, on heating to about 200°, turned colorless and decomposed at 301–304°. Uv (MeOH) λ max nm (log ε) 219 (4.54) 236 sh (4.37), 285 (4.49), 319 sh (4.08), 345 (4.21), 412 sh (3.16), 501 (3.34); ir (KBr) ν max cm^{-1} 3420, 1640, 1635, 1620, 1502, 1475, 1340, 1270, 1253, 1210, 1040; eims *m/z* (rel. int. %) $[M]^+$ 378 (5), 377 (7), 363 (50), 348 (44), 262 (26), 182 (85), 174 (55), 159 (39), 145 (90), 131 (100), 117 (47), 102 (44); ^1H nmr see Table 1.

10-Hydroxychelerythrine chloride [**7a**].—Orange crystals from MeOH/concentrated HCl: mp 244–246°; uv (MeOH) λ max nm (log ε) 229 (4.50), 279 (4.55), 336 (4.24), 349 sh (4.15), 410 sh (2.97), 474 (3.12); ir (KBr) ν max cm^{-1} 3420, 1592, 1490, 1470, 1398, 1355, 1265, 1250, 1212, 1125, 1030, 929, 877; eims *m/z* (rel. int. %) $[M]^+$ 364 (13), 363 (12), 349 (50), 334 (54), 175 (54), 153 (100); ^1H nmr see Table 1.

10-Hydroxydihydrosanguinarine [**2b**].—Colorless crystals from $CHCl_3$/MeOH: mp 208–211° (dec); uv (MeOH) λ max nm (log ε) 224 (4.57), 277 (4.49), 304 sh (4.02), 338 (4.25), 351 (4.20); ir (KBr) ν max cm^{-1} 3500, 1642, 1620, 1460, 1432, 1273, 1240, 1212, 1188, 1035, 855; eims *m/z* (rel. int. %) $[M]^+$ 349 (100), 348 (51), 334 (13), 175 (49), 167 (27), 149 (54), 146 (27), 145 (29), 138 (34), 131 (30), 117 (44), 102 (44); ^1H nmr see Table 2.

REDUCTION OF **2a**, **4a**, AND **7a** WITH $NaBH_4$.—To an ice-cooled and stirring solution of **2a** (12.0 mg) in MeOH (3 ml) was added $NaBH_4$ (5 mg) in portions. The mixture was stirred for a further 30 min. After dilution with H_2O, the reaction mixture was extracted with C_6H_6 (20 ml × 5). The washed and dried organic layer was concentrated to give a residue (8.5 mg), which was purified by preparative tlc [*n*-hexane–EtOAc (5:2)], yielding **2b** (7.2 mg). This compound was identical to the isolate from the cultured cells. In similar manner, **4a** and **7a** were reduced with $NaBH_4$ to afford **4b** and **7b**, respectively.

12-Hydroxydihydrochelirubine [**4b**].—Colorless crystals from $CHCl_3$/MeOH: mp 219–221° (dec); uv (MeOH) λ max nm (log ε) 218 (4.61), 237 sh (4.45), 285 (4.57), 315 (4.18), 338 (4.21); ir (KBr) ν max cm^{-1} 3415, 1615, 1493, 1467, 1450, 1285, 1242, 1225, 1108, 1055, 1030; eims *m/z* (rel. int. %) $[M]^+$ 379 (71), 378 (21), 364 (38), 349 (19), 190 (100), 161 (43), 153 (77), 139 (43); ^1H nmr see Table 2.

10-Hydroxydihydrochlerythrine [**7b**].—Colorless crystals from $CHCl_3$/MeOH: mp 191–193°; uv (MeOH) λ max nm (log ε) 227 (4.53), 277 (4.54), 326 (4.18), 332 (4.18), 348 sh (3.97); ir ($CHCl_3$) ν max cm^{-1} 1620; eims *m/z* (rel. int. %) $[M]^+$ 365 (100), 364 (43), 350 (18), 349 (24), 335 (18), 320 (11), 183 (36), 175 (45), 168 (45), 153 (31), 146 (32), 139 (26), 138 (26), 125 (30), 111 (36), 102 (31); ^1H nmr see Table 2.

METHYLATION OF **2b**, **4b**, AND **7b**.—A solution of **2b** (3.6 mg) in MeOH (2 ml) was treated with CH_2N_2/Et_2O and kept overnight at 4°. The reaction mixture was concentrated in vacuo, and the resulting residue was subjected to preparative tlc [*n*-hexane–Et_2O (5:1)], giving rise to **3b** (1.9 mg). The product was identified with an authentic sample. In the similar manner, **4b** and **7b** were methylated to afford **5b** and **8b**, respectively.

DETECTION OF **2b** AND **4b** IN 48,000*g* PELLET FROM THE CELL SUSPENSION CULTURES OF *E. CALIFORNICA*.—An aqueous solution of baker's yeast elicitor (80 mg/liter medium) was applied 4 days after inoculation of cells into fresh LS medium. The fresh cells were harvested after 20 h of agitation, frozen in liquid N_2, and ground gently in a cold mortar in 2 volumes of 0.1 M tricine-NaOH buffer (pH 7.5) containing 250 mM sucrose and 5 mM thioglycolic acid. The slurry was squeezed through four layers of cheesecloth and the filtrate centrifuged at 3000 *g* for 5 min. The 3000 *g* supernatant was treated with 1 M $MgCl_2$ (final concentration 50 mM) and the resulting precipitate centrifuged at 48,000*g* for 15 min. The pellet was resuspended in 0.1 M tricine-NaOH buffer (pH 7.5) containing 5 mM thioglycolic acid and resedimented. The pellet (containing largely microsomes) was again resuspended in the same buffer as above, and 200 μl of the resultant suspension was mixed with 800 μl of MeOH for 1 h and subsequently centrifuged for 5 min (Eppendorf systems). The supernatant was employed for hplc analysis. The alkaloids contained in this fraction were identified by their retention times. Large scale preparation of a 48,000*g* pellet led to the isolation of the two major compounds by preparative tlc [C_6H_6-Et_2O (9:1)]; these compounds were characterized by standard procedures (co-tlc, eims, ^1H nmr) and identified as **2b** and **4b**. Protein was determined according to Bradford (15).

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LITERATURE CITED

1. G.A. Cordell, "Introduction to Alkaloids—A Biogenetic Approach," Wiley-Interscience, New York, 1981, pp. 511–513.
2. N. Takao, M. Kamigauchi, and M. Okada, *Helv. Chim. Acta*, **66**, 473 (1983).
3. H.-M. Schumacher, H. Gundlach, F. Fiedler, and M.H. Zenk, *Plant Cell Rep.*, **6**, 410 (1987).
4. J. Berlin, E. Forche, V. Wray, J. Hammer, and W. Hösel, *Z. Naturforsch.*, **38C**, 346 (1983).
5. M.A. Collinge and P.E. Brodelius, *Phytochemistry*, **28**, 1101 (1989).
6. T. Tanahashi and M.H. Zenk, *Tetrahedron Lett.*, **29**, 5625 (1988).
7. L. Hruban, F. Santavý, and S. Hegerová, *Collect. Czech. Chem. Commun.*, **32**, 3414 (1970).
8. H.-M. Schumacher and M.H. Zenk, *Plant Cell. Rep.*, **7**, 43 (1988).
9. U. Eilert, W.G.W. Kurz, and F. Constabel, *J. Plant Physiol.*, **119**, 65 (1985).
10. J.L. Dzink and S.S. Socransky, *Antimicrob. Agents Chemother.*, **27**, 663 (1985).
11. G. Blaskó, G.A. Cordell, S. Bhamaravati, and C.W.W. Beecher, *Heterocycles*, **27**, 911 (1988).
12. N. Takao, M. Kamigauchi, M. Sugiura, I. Ninomiya, O. Miyata, and T. Naito, *Heterocycles*, **16**, 221 (1981).
13. H. Ishii, F. Ishikawa, T. Watanabe, Y. Ichikawa, and E. Kawanabe, *J. Chem. Soc., Perkin Trans. 1*, 2283 (1984).
14. H. Linsmaier and F. Skoog, *Physiol. Plant.*, **18**, 100 (1965).
15. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).

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