Journal of Natural Products
Vol. 51, No. 3, pp. 532-536, May-Jun 1988

PAPAVERINE BIOTRANSFORMATION IN PLANT CELL SUSPENSION CULTURES

P. DORISSE, J. GLEYE, P. LOISEAU, P. PUIG, A.M. EDY, and M. HENRY*

Faculté de Pharmacie, 31 Allées Jules Guesde, 31000 Toulouse, France

ABSTRACT.—The biotransformation of papaverine HCl was studied using cell suspensions of *Saponaria officinalis* and *Glycyrrhiza glabra*. With *S. officinalis* no product of bioconversion and no toxic effect were detected even when papaverine HCl was added at a high level (1250 mg/liter). *G. glabra* showed sensitivity above 250 mg/liter of papaverine HCl and afforded different products of oxidation and demethylation. Only one of them, papaverinol, was produced in relatively high amount (31.5%) as quantified by radioactive labeling of the papaverine.

Cell cultures of *Silene alba* and *Vinca minor* have been reported to convert papaverine hydrochloride (Pap.HCl) into products of demethylation or oxidation (1–5). As an extension of these results we describe here the biotransformation of papaverine by cell suspensions of *Glycyrrhiza glabra* L. var. *typica* Reg. and Hed. (Leguminoseae) and *Saponaria officinalis* L. (Caryophyllaceae).

EXPERIMENTAL

PLANT MATERIAL.—*S. officinalis* shoots were harvested from voucher plants growing in the Museum National d' Histoire Naturelle, Paris, France. The shoots were surface-sterilized in 5% sodium hypochlorite (10 min). Plants of *G. glabra* were kindly supplied by Desmarest of the Pernod Ricard Research Center, Creteil, France.

TISSUE CULTURE.—Primary calli of *S. officinalis* were originated in 1978 from stems of shoots. They were induced at 25 ± 1° under fluorescent light (2000 lux, 12 h of photoperiod) on Nitsch and Nitsch's medium (6) containing 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/liter), adenine (2 mg/liter), kinetin (1 mg/liter), sucrose (20 g/liter), and agar (8 g/liter). The pH was adjusted to 5.7 before autoclaving at 120° for 20 min. These calli were then subcultured every 3 weeks under the same environmental conditions on Murashige and Skoog's medium (7) containing 2,4-D (0.1 mg/liter), adenine (1 mg/liter), kinetin (1 mg/liter), glucose (30 g/liter), and agar (8 g/liter) at pH 5.7. The strain studied here was not chlorophyllous and had a pale yellow color after one year of stabilization. Primary calli of *G. glabra* were induced from stems of plants since January 1984, and subcultured under the same environmental conditions with the same medium as used for subculture of *S. officinalis* calli with only one difference: 2,4-D was replaced by α-naphthalene acetic acid (NAA, 0.01 mg/liter). The strain used for this study was chlorophyllous and was morphologically stable after 6 months of subculture.

Cell suspension cultures were carried out in liquid medium with the same composition (330 ml in a 1-liter Erlenmeyer flask) on a gyratory shaker (120 rpm for *S. officinalis*, 80 rpm for *G. glabra*) at 25 ± 1° (in the dark for *S. officinalis*; under the previous environmental conditions for *G. glabra*) and subcultured (every week for *S. officinalis*, every 2 weeks for *G. glabra*) during 6 months prior to any experimentation.

GROWTH MEASUREMENT.—Growth of cell suspensions was measured by fresh or dry wt determination and confirmed by the cell number after callus dissociation with Cr_2O_3 10% at 70° during 10 min (Henshaw's method modified) (8).

TOXICITY ESTIMATION.—To estimate the toxicity of Pap.HCl upon the cells, different concentrations of sterile Pap.HCl were added to 250-ml flasks of cell suspension (12% of packed cell volume each) at the beginning of the batch culture. Cells and medium were harvested at the stationary phase (day 9 or 11 for *S. officinalis*, day 21 for *G. glabra*, (Table 1) and separated by filtering prior growth measurement.

ANALYSIS OF STANDARDS.—Pap.HCl was purchased from Boyer, France, and purified by cc before use. Purification was performed on Si gel elution with a $CHCl_3$/MeOH gradient. Pap.HCl was crystallized from MeOH. Papaveraldine was purchased from Extrasynthese, France.

GENERAL CHEMICAL PROCEDURES.—Uv spectra were recorded on UNICAM SP 800 spectrophotometer. Mass spectra were determined on an ABI MS 902 instrument. Ir spectra were determined on a Perkin-Elmer 983-G instrument. ^{1}H-nmr spectra were determined on a Varian T-60 (60 MHz) instrument or on a Brucker (90 MHz) instrument with TMS as the internal standard; chemical shifts are re-

TABLE 1. Papaverine Toxicity on the Growth of *Saponaria officinalis* and *Glycyrrhiza glabra* Cell Suspensions.

Cell suspension	Pap.HCl content (mg/liter)	Inoculum (dry wt g/liter)	Maximal dry wt at the stationary phase (g/liter)	Specific growth rate (h^{-1})	Fresh wt content at day 21 (g/liter)
S. officinalis[a]	0	3.6	17 on 9th day	8.0×10^{-3}	
	50	3.6	20 on 9th day	8.2×10^{-3}	
	250	3.6	18.5 on 9th day	8.2×10^{-3}	
	1250	3.6	18 on 11th day	6.2×10^{-3}	
G. glabra[b]	0				500
	100				450
	200				265
	300				210
	400				160
	500				145
	1000				85

[a]For each Pap.HCl concentration, a batch culture pattern was carried out. Growth was measured as dry wt content.
[b]For each Pap.HCl concentration, 100 ml of 3-week-old cell suspension was analyzed for fresh weight content to estimate the toxicity level (inoculum 40 mg/liter).

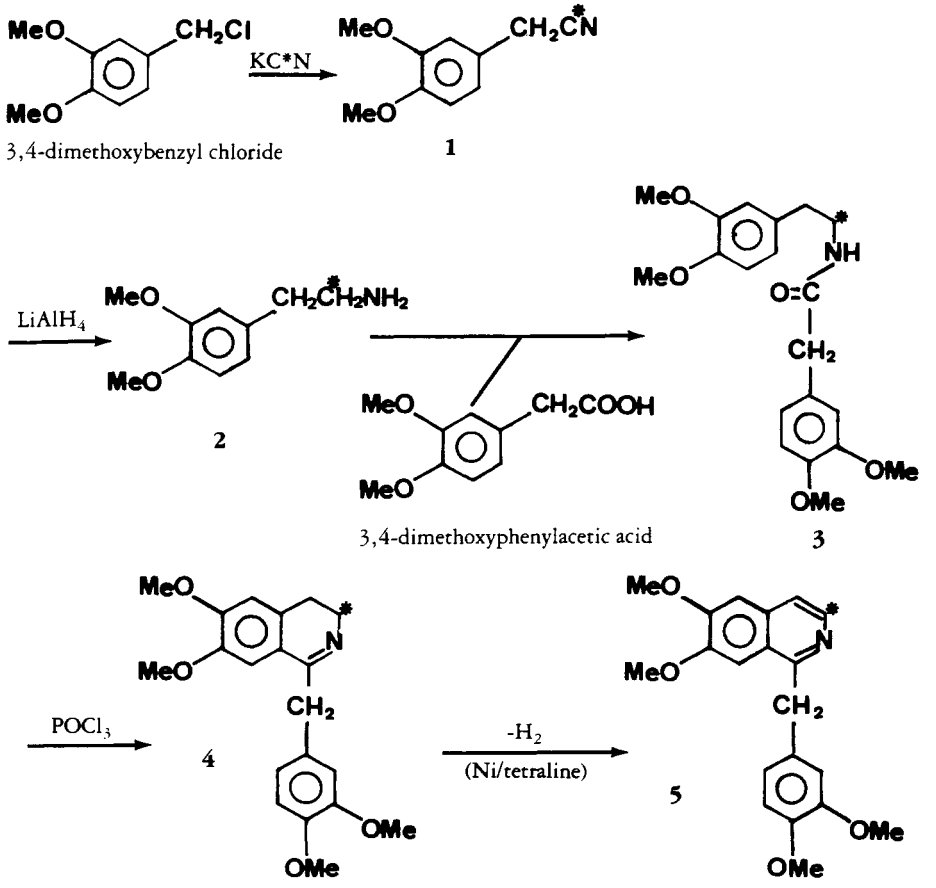

FIGURE 1. Outline of synthesis of 3-^{14}C-labelled papaverine.

ported in ppm (δ). An LKB WALLAC Rackbetta 1215 liquid scintillation counter was used to measure radioactivity. The ^{14}C determinations were made with Ready Solv MP Beckman scintillation fluid. Si gel (Merck type 60, 70–230 mesh) and Amberlite XAD-2 (Rohm and Hass) were used for cc, and precoated Si gel plates (Merck, 60F-254, 0.25 and 2 mm) were employed for analytical and preparative tlc, respectively. Hplc was performed on a Waters Associates Pump model 510 with an RP-8 (Merck, 10 μm) column (300 × 3.9 mm) for analytical determinations. Pap.HCl was quantified at 280 nm with a Waters Associates Lambda-Max Model 481 LC Spectrophotometer after elution with MeOH-H_2O-diethylamine (65:35:0.05) at 1.5 ml/min flow.

SYNTHESIS OF 3-^{14}C PAPAVERINE.—*3,4-dimethoxyphenylacetonitrile 1-^{14}C* [**1**].—3,4-Dimethoxybenzylchloride (9) (4.15 g, 0.022 mol) was dissolved in 30 ml of anhydrous DMF; Na ^{14}C-cyanide (CEA, Saclay) (0.1 mg) with specific activity 47.7 mCi/mmol and KCN (1.45 g, 0.022 mol) were added. The mixture was stirred at 70° for 12 h; 1.45 g of KCN was added again and the mixture stirred at 120° for 12 h. After evaporation under vacuum to dryness, the residue was extracted with $CHCl_3$. The extracts gave 3.1 g of 3,4-dimethoxyphenylacetonitrile 1-^{14}C [**1**] (yield 79%, mp 64°) that contained 86 μCi: ir ν max (KBr) 2248 (C N); ^1H nmr ($CDCl_3$) δ 7.10 (3H, m, Ar), 4.00 (6H, s, OMe), 3.78 (2H, s, CH_2).

2-(3,4-dimethoxyphenyl)ethylamine 1-^{14}C [**2**].—To 1 g of $LiAlH_4$, suspended in 50 ml of anhydrous Et_2O, was added dropwise 3.0 g (0.017 mol) of **1** in 50 ml of Et_2O. The mixture was heated under reflux for 3 h. Dilute NaOH (2 N) was added and the mixture extracted with Et_2O. After drying, the solvent was distilled off to give 2.9 g of **2** (yield: 94%, nD^{25} = 1.4682) which contained 76 μCi.

N-*(3,4-dimethoxyphenyl)ethyl 3,4-dimethoxyphenylacetamide-^{14}C* [**3**].—3,4-dimethoxyphenylacetic acid (540 mg, 2.75 mmol) was added to 500 mg (2.76 mmol) of **2** and 20 ml of dry xylene. The mixture was heated under reflux for 4 h. Most of the solvent was removed under vacuum to give a cream-colored residue. This was recrystallized from EtOAc to give 710 mg of **3** (yield 72%, mp 125°) which contained 9.37 μCi.

3,4-dihydropapaverine 3-^{14}C [**4**].—Compound **3** (700 mg) was dissolved in 10 ml of anhydrous C_6H_6, and 0.5 ml of $POCl_3$ was added. The mixture was heated under reflux for 40 min under N_2 atmosphere. After cooling, 5 ml of H_2O was added, and the mixture was heated for 15 min at 90°. The aqueous layer was made alkaline with dilute aqueous Na_2CO_3 and extracted with CH_2Cl_2. After drying and removing the solvent, we obtained 610 mg of **4** (yield 92%, oil).

Papaverine 3-^{14}C [**5**].—Tetralin (10 ml), 600 mg of crude **4**, and 0.9 g of 10% Pd/C were heated at 160–165° for 1.5 h under N_2 atmosphere. During this, dry Me_2CO was added dropwise from time to time and distilled off. After cooling, MeOH was added and the mixture filtrated; removing the solvent under vacuum gave 450 mg of crude product (yield 75%) which was purified by chromatography on a preparative Si gel plate ($CHCl_3$-Me_2CO, 70:30). The fraction which contained papaverine was eluted to give 200 mg of **5** (yield 34%, mp 147°) with 2.7 μCi (13.5 μCi/g, 4.57 μCi/mmol). For further experiments, papaverine was used as the hydroxhloride (prepared from **5** and HCl in Et_2O).

ISOLATION OF ALKALOIDS.—Freeze-dried tissues and media were extracted three times with MeOH for 24 h. After evaporating the extracts to dryness, the residues were dissolved in MeOH-H_2O (65:35). After centrifugation, the supernatant was analyzed by tlc on Si gel 60F 254. $CHCl_3$-Me_2CO (70:30) was used as developing solvent. The unknown compounds were compared with standard alkaloids (papaverine and papaveraldine). Spots were observed in 254 nm and 366 nm light and after spraying the plates with Dragendorff's reagent. Papaverine contents were determined by hplc. For the determination of the structure of the by-products, the crude extracts obtained from the suspension medium and from the cells were purified by cc on Amberlite XAD-2 with H_2O and a progressively higher percentage of MeOH as eluent. The 100% MeOH fraction was purified on Si-gel 60 with CH_2Cl_2 and a progressively higher percentage of Me_2CO as eluent. Three alkaloids were isolated by preparative tlc on Si gel 60 plates with $CHCl_3$-Me_2CO (70:30) (eluting system A) or with *n*-BuOH saturated with H_2O-MeOH (90:10) (eluting system B). These compounds were comparable in all the spectral and physicochemical properties with that obtained from authentic standards or data given in the literature.

One compound was identical to papaveraldine (4,10): R_f 0.88 (A); uv λ max (EtOH) 240, 280, 326; ir ν max (KBr) 1655 cm^{-1} (C=O); eims (70 eV) *m/z* $[M]^+$ 353 (50%), $[M-Me]^+$ 338 (55%), $[M-CO]^+$ 325 (47.5%), $[M-H-CO$ or $M-MeO]^+$ 324 (80%), $[M-MeO]^+$ 322 (75%), $[M-CO-Me]^+$ 310 (48%), $[M-CO-MeO]^+$ 294 (43%), $[310-MeO]^+$ 279 (38%), 165 (100%) (benzylic fragmentation).

A second compound was identical to papaverinol (10): R_f 0.57 (B); uv λ max (EtOH) 240, 280; ir ν max (KBr) 3425 cm^{-1} (OH); eims (70 eV) *m/z* $[M]^+$ 355 (37%), $[M-OH]^+$ 340 (8.5%), $[M-Me]^+$ 338 (25%), $[M-MeO]^+$ 324 (20.5%), $[M-3Me]^+$ 308 (29%), $[M-2MeO]^+$ 293 (15.5%), 218 (40.5%) benzyl fragmentation), 202 (25%) (benzyl fragmentation), 189 (50%) (benzyl fragmentation), 151 (100%) (benzyl fragmentation); ^1H nmr ($CDCl_3$, 90 MHz) δ 8.40 (1H, d, H-3, J = 5.57), 7.51 (1H.

d, H-4, $J = 5.57$), 7.10 (1H, d, H-5', $J = 4.40$), 7.07 (1H, s, H-2'), 6.87–6.72 (3H, m, H-5, H-8, H-6'), 6.15 (1H, s, H-COH), 3.99 (3H, s, OMe), 3.82 (3H, s, OMe), 3.81 (3H, s, OMe), 3.76 (3H, s, OMe).

The last compound was identical to monodemethylpapaverine (3): R_f 0.30 (A); eims (70 eV) *m/z* $[M]^+$ 325 (36.5%), $[M-1]^+$ 324 (100%), $[M-Me]^+$ 310 (76.6%), $[M-MeO]^+$ 294 (24.9%), $[M-MeO]^+$ 279 (11.5%), 151 (7.7%) (benzylic fragmentation).

BIOTRANSFORMATION OF LABELED PAPAVERINE BY *G. GLABRA* CELL SUSPENSION.—Labeled Pap.HCl (250 mg/liter, 5.07 μCi/mmol) was added to the *G. glabra* cell suspension at the beginning of the batch culture. The cell suspension was incubated as described for 21 days and harvested by filtration. The alkaloid extraction was carried out as described earlier. Quantitative analysis of the Pap.HCl biotransformation was performed by radioactive determination of the different by-products after preparative tlc separation (eluting system A for compounds **6** and **8**, eluting system B for the product **7**).

RESULTS AND CONCLUSION

Pap.HCl did not show any toxic effects on cells of *S. officinalis*, even at a level of 1250 mg/liter (Table 1). However, *G. glabra* cells exhibited sensitivity, with a level of 100 mg/liter being slightly toxic (Table 1). The ID_{50} was about 250 mg/liter and was the dose chosen for the following studies with both cell suspensions.

When Pap.HCl was added to the cell suspension of *S. officinialis* at the beginning of the batch culture, no product of bioconversion was detected by tlc either in the cells or in the medium even during the stationary phase and even when 1250 mg/liter was added to the cell suspension. *G. glabra* afforded different by-products as indicated by analytical tlc.

The Pap.HCl content over a period of time was monitored and the results shown in Table 2. In the medium, it decreased continuously for several days after the cell suspension inoculation, both in the case of *C. officinalis* and G. glabra. An increase could be observed only for *S. officinalis* at the end of the stationary phase. In the cells the Pap.HCl content increased continuously for both cell suspensions, but this increase was higher

TABLE 2. Variation of the Papaverine Content in the Cells and the Culture Medium of Two Cell Suspensions During Growth in a Batch Culture.[a]

Saponaria officinalis	Days								
	0	2	4	7	9	11	14	16	18
Cell growth (fresh wt g/liter)									
Without Pap.HCl	50	64	130	440	480	380	380	350	260
With 250 mg/liter Pap.HCl	50	60	135	380	440	400	370	300	240
Pap.HCl content (mg/liter)									
Medium	204	174	154	123	81	64	70	116	115
Cells	22	12	43	83	119	134	132	116	90
Cells + Medium	226	186	197	206	200	198	202	232	204

Glycyrrhiza glabra	Days					
	0	7	14	21	28	35
Cell growth (fresh wt g/liter)						
Without Pap.HCl	55	99	265	400	402	405
With 250 mg/liter Pap.HCl	55	85	122	225	265	290
Pap.HCl content (mg/liter)						
Medium	212	186	167	96	58	68
Cells	31	41	46	61	76	63
Cells + Medium	243	227	213	157	134	131

[a]Pap.HCl (250 mg/liter) was added to the two cell suspensions at the beginning of the culture. Cell suspensions (100 ml) were harvested by filtration on the days indicated. The fresh wt and the papaverine content of the cells and the medium were determined as described in Experimental section. Each determination was made in triplicate.

with *S. officinalis*. A rather constant level in the cell suspension of *S. officinalis* was noted when the medium and cell contents were considered together. On the contrary, in *G. glabra* cell suspension the total Pap.HCl content decreased continuously to reach about 100 mg/liter during the stationary phase. After 21 days of the batch culture, labelled Pap.HCl was essentially biotransformed to papaverinol (31.5%), papaveraldine (4%), and demethylpapaverine (1%). The latter product was too minor to allow the identification of the position of the methoxy group by ^{1}H-nmr spectrometry. About 60% of the starting papaverine remained radioactive. There was no other radioactive product in the cells or medium. Pap.HCl dissolved in fresh medium or in a cell suspension killed after autoclaving did not give any by-products when it was maintained in the same conditions. The products described here were, thus, definitely obtained by biotransformation of the papaverine and not by degradation of this molecule (4). The cell suspension of *G. glabra* was able to biotransform, in rather good yield (31.5%), exogenous papaverine to the hydroxylated compound, papaverinol. This by-product was not noted in previous biotransformations with cell suspensions or callus cultures of different species (1–5). Quantification of the different by-products was carried out by radioactive labeling of the papaverine. Although labeling of this compound had previously been done (11), the method employed here was different from the previous work. Use of this labeled compound in the experiments indicated that no other products of biotransformation were obtained from papaverine.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministère de la Recherche et de la Technologie (no. 417 84).

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Received 9 November 1987