PROTOANEMONIN DETECTION IN *CALTHA PALUSTRIS*

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Caltha palustris L. (Ranunculaceae) or marsh-marigold, a perennial herb commonly distributed in the Northern hemisphere, is well known for its toxicity for cattle, horses, and man. In Italy, this plant frequently occurs in the marshes and wet meadows of the Po Valley and in the water pools of mountain areas. The toxicity of marsh-marigold is often attributed to its protoanemonin content (1-4); however, several reports refer to the absence of this vesicant principle (5,6). In the present paper, we report for the first time the separation and quantitation of protoanemonin in *C. palustris* by a selective hplc procedure.

According to our previous experience, protoanemonin was extracted by a micro steam distillation apparatus. The extracts were either directly injected into the hplc system or kept at 4° for a time not longer than 1 week. Normal-phase (NP) or reversed-phase (RP) hplc could be used (7); however, NP-hplc is preferable because it reduces the possible interference of other compounds with protoanemonin. In *C. palustris* protoanemonin was obtained by a micropreparative-scale separation on an analytical column in the normal phase mode as suggested by Verzele *et al.* (8). The eluate corresponding to the protoanemonin peak (K' 3.6) was collected and immediately identified by comparing chromatographic, spectrophotometric, and colorimetric parameters with those of the standard, following the identification procedure previously reported (7). Six marsh-marigold populations growing in the Po Valley were analyzed for their protoanemonin content. All of the plants examined contained protoanemonin with a mean content of 0.26 μg/g wet wt. In other Ranunculaceae protoanemonin content is very high. For instance, in *Helleborus niger* the compound reaches 5,820 μg/g wet wt. These results confirm the presence of protoanemonin in marsh-marigold. It is unlikely, however, for such a small amount of protoanemonin to be the only toxic and irritant agent in *C. palustris*. Therefore, possible cases of marsh-marigold poisoning are probably explained by the presence of other compounds such as pyrrolizidine and aporphine alkaloids that are known to exist in other toxic *Caltha* species (9). The small amount of protoanemonin may be considered as an irritant cofactor. The demonstration of protoanemonin in *C. palustris*, a member of an ancestral genus previously considered to lack protoanemonin (5,6), may be of relevance in chemotaxonomic studies of systematic relationships within the Ranunculaceae. If future studies confirm the presence of protoanemonin in other *Caltha* species, the homogeneity of the Helleboreae within the Ranunculaceae will be consolidated. Further research on the significance of protoanemonin and its possible role as chemical marker is in progress.

EXPERIMENTAL

PLANT MATERIAL.—The plant material was collected during the summer of 1985, in different marshes of the eastern Po Valley. Leaf samples of flowering plants were immediately put into plastic bags, weighed, and frozen within 2 h from collection. The plants were identified as *C. palustris* subsp. *palustris* and voucher specimens are kept in the Herbarium of the Institute of Botany, University of Ferrara, Italy.

STANDARD.—A standard sample of protoanemonin was obtained by *n*-hexane extraction of the steam distillate of *Helleborus niger*.

HPLC CONDITIONS.—The apparatus used was as follows: hplc, Varian Model 5020; Columns, Hibar Lichrosorbr RP-18 (10 μm) (30×0.40 cm i.d.) with a Varian MCH-10 guard column, Hibar Lichrosorb Si 60 (5 μm) (25×0.40 cm i.d.) with a Varian Si 10 (10 μm) (3×0.46 cm i.d.) guard column; uv-vis spectrophotometer, Perkin-Elmer Model 554.

The RP mobile phase was MeCN-H_2O (20:80) at 2.5 ml/min and the NP mobile phase was *n*-hexane-Et_2O-CH_2Cl_2-$CHCl_3$ (70:10:10:10) at 2.0 ml/min. Standard curves for protoanemonin were linear in the range of 0.5-10 μg/ml.

SPECTROSCOPIC CONDITIONS.—Spectrophotometric characteristics of protoanemonin and its reaction products were determined in the 220-300 and 440-600 nm ranges using a 60 μl cell with a 1 cm lightpath. Full details of the spectroscopic identification are available upon request.

ANALYSIS PROCEDURES.—The steam distillate was obtained from 10 g of fresh leaves. After extraction by *n*-hexane until exhaustion, it was dehydrated, purified, and concentrated by flowing into small glass columns, prepared in our laboratory, containing Na_2SO_4 (25×0.90 cm i.d.) and silica gel 60 (40 μm) (5×0.60 cm i.d.).

ACKNOWLEDGMENTS

This work was supported by a research grant from Consiglio Nazionale delle Richerche (CNR) and Ministero della Pubblica Istruzione of Italy.

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Received 5 June 1986

PRESENCE OF BORNYL *p*-COUMARATE IN THE ROOTS OF *EUPATORIUM DELTOIDEUM*

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Previous chemical work on the aerial parts of *Eupatorium deltoideum* Jacq. (Compositae) revealed the presence of sesquiterpene lactones of the guainolide, germacranolide, and heliangolide types (1). We report here the isolation and identification of bornyl *p*-coumarate from the roots of *E. deltoideum*.

This seems the first time that bornyl *p*-coumarate has been isolated from a *Eupatorium* species, although it was previously found in *Verbesina rupestris* (2). The structure became evident by comparing its ^{13}C-nmr spectrum with those of bornyl acetate (3) and *p*-coumaric acid (4).

EXPERIMENTAL

PLANT MATERIAL.—*E. deltoideum* was collected in September 1985 along the México-Puebla Highway (33 km). A voucher specimen (1) (Quijano 26) was deposited at Herbario Nacional del Instituto de Biología, Universidad Nacional Autónoma de México.

EXTRACTION, SEPARATION, AND IDENTIFICATION.—Powdered, dried roots of *E. deltoideum* (2 kg) were extracted with EtOAc (4 liters). The extract was concentrated under reduced pressure and chromatographed over Si gel. The fractions eluted with hexane-EtOAc (8:2) were recrystallized from $CHCl_3$/hexane to afford 125 mg of bornyl *p*-coumarate, mp 146-148° [lit. (2) mp 153-154° ($MeOH/H_2O$)]; $[\alpha]^{25}D-33.2°$ (*c*, 5 $CHCl_3$); uv (MeOH) λ max nm (log ϵ) 213 (4.00), 228 (4.02), 313 (4.32); ir ($CHCl_3$) ν max cm^{-1} 3590, 3380, 3015, 1694, 1635, 1606; ^{1}H-nmr (60 MHz, C_6D_6) δ 0.76 (s, 3H, Me), 0.80 (s, 3H, Me), 0.92 (s, 3H, Me), 5.30 (dd, J=10, 4 Hz, 1H, H-2), 6.43 (d, J=16 Hz, 1H, H-2'), 6.80 (d, J=8 Hz, 2H, H-5', and H-9'), 7.16 (d, J=8 Hz, 2H, H-6', and H-8'), 7.93 (d, J=16 Hz, 1H, H-3'); ^{13}C nmr (25.2 MHz, $CDCl_3$) δ 168.7 (s, C-1'), 158.5 (s, C-7'), 144.8 (d, C-3'), 129.9 (d, C-5', and C-9'), 126.4 (s, C-4'), 115.8 (d, C-6', and C-8'), 115.1 (d, C-2'), 80.4 (d, C-2), 48.9 (s, C-1), 47.8 (s, C-7), 44.9 (d, C-4), 36.8 (t, C-3), 28.0 (t, C-5), 27.2 (t, C-6), 19.7 (q, C-8), 18.8 (q, C-9), 13.6 (q, C-10).