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ISOLATION AND CHARACTERIZATION OF CHELIDONIC ACID FROM *SORGHUM VULGARE**

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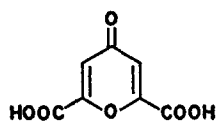
Abstract—Chelidonic acid (4-pyrone-2,6-dicarboxylic acid) (I) was isolated from etiolated *Sorghum vulgare* seedlings and characterized by IR, MS and UV spectra, elementary analyses and chromatographic properties The chelidonic acid concentration decreased about 3-fold from 1 7 μmoles/g fresh wt in 2-day-old seedlings to 0 5 μmoles/g fresh wt in 4-day-old etiolated seedlings Although ^{14}C from exogenous L-tyrosine became incorporated into chelidonic acid most of the chelidonate synthesized was derived from endogenous substrate

INTRODUCTION

DURING investigations on the biosynthesis of dhurrin in etiolated sorghum seedlings the presence of a substance with an absorption maximum near 270 nm was noted [1] The absorption spectrum suggested it might be a phenolic substance. Because significant quantities were present, it was of interest to characterize this substance and to determine if it was a precursor of dhurrin or related to tyrosine metabolism in any other manner.

This paper describes the isolation of this substance from *Sorghum vulgare* seedlings and its characterization as chelidonic acid (4-pyrone-2,6-dicarboxylic acid, I) by spectral methods and elemental analyses. Although chelidonic acid was reported previously as a constituent of maize,[2] and other species of grasses, the identification was based solely on chromatographic and UV spectral properties.[3]



(I) Chelidonic acid

RESULTS AND DISCUSSION

Approximately 10 g of etiolated sorghum seedlings were extracted with 3 vol. (w/v) of 0·3 M perchloric acid as described in the Experimental. After removal of cell debris and perchlorate as the potassium salt, the supernatant was adjusted to pH 5–6 and transferred to a column containing Dowex-1-Br The resin was irrigated with a solution containing up to 0·7 M KBr when several substances absorbing at 254 nm were eluted from the column.

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[1] J E GANDER, *Fed Proc* **19**, 3 (1960)
[2] J CHERRY and R H HAGEMAN, *Plant Physiol* **35**, 343 (1960)
[3] M R ATKINSON and G ECKERMANN, *Austral J Biol Sci* **18**, 437 (1965)

The fractions eluting between 400 and 450 ml were pooled, extracted with ether for 2 days at neutral pH and the ether extract discarded The aqueous phase was acidified to approximately pH 1 with HCl and extracted continuously for 5 days The ether was removed and the residue was dissolved in water Samples from this solution had the same chromatographic and colour properties as authentic chelidonic acid

Chromatography on Dowex-1-bromide did not cleanly separate chelidonic acid from dhurrin, the major nonprotein phenolic substance in etiolated sorghum seedlings, and a portion of the dhurrin and *p*-hydroxybenzaldehyde was extracted into the acidic ether phase. A rapid separation of chelidonic acid from dhurrin is achieved by applying the neutral perchlorate extract to a column containing PVP and irrigating the column with water This procedure results in minimal destruction of dhurrin and allows the recovery of both dhurrin and chelidonic acid Protein is the first major substance absorbing at 254 nm eluted, chelidonate and tyrosine are both eluted together, and dhurrin is eluted in later fractions *p*-Hydroxybenzaldehyde remains on the column during this treatment. Fractions containing chelidonate were pooled, adjusted to pH 1 and extracted continuously with ether for 5 days Chelidonic acid was obtained in the ether extract A negligible quantity of tyrosine was extracted by this procedure

In order to further characterize the substance, a large scale isolation and purification was carried out on 1590 g of 9-day-old etiolated seedlings as described in the Experimental Chelidonic acid so obtained crystallized as the monohydrate and a yield of approximately 45% was obtained based on a chelidonic acid concentration of 0·5 μmoles/g fresh wt in the seedlings Elemental analyses gave an empirical formula of $C_7H_6O_7$ (C, 42·1%, H, 2·36%, O, 53·7%, P, 0·0%, residue 0·4%) a value consistent with chelidonic acid monohydrate The molecular weight, estimated by potentiometric titration, was 101 per carboxyl group

Mass spectral analysis showed a molecular ion of mass 184, peaks with approximately the same intensity at 156 (M—28) and 140 (M—44) which corresponds to the loss of carbon monoxide and carbon dioxide respectively, a major peak of 112 (M—72) which could correspond to loss of both carbon dioxide and carbon monoxide and a small peak, 5% of the parent ion, at 167 (M—17) corresponding to the loss of hydroxyl ion. The mass spectrum was identical to that of authentic chelidonic acid

The UV spectrum in water and 1 N KOH were also identical to authentic chelidonic acid The IR spectrum in a Nujol mull was nearly identical to that published by Sadtler[4] for chelidonic acid in a KBr wafer, the only difference being in the 3500–3400 cm^{-1} region This probably represents the difference in interaction between the carboxyl groups in these two supporting agents All this evidence supports our conclusion that sorghum seedlings contain chelidonic acid.

We have reported recently[5] that dhurrin turns over rapidly in sorghum seedlings and have in addition shown that its concentration gradually decreases as the seedling ages Figure 1 shows the relationship between the quantity of dhurrin and chelidonic acid in etiolated sorghum seedlings over a period of time before the seedlings leaf out There is an approximately 3-fold decrease in chelidonate concentration between the second and fourth day after germination of seedlings, and this decrease parallels the decrease in dhurrin although dhurrin is present at 40-fold higher concentration This evidence, when coupled with our earlier observations that dhurrin is metabolized rapidly in sorghum seedlings,[5] eliminates chelidonic acid as a major inert end product of dhurrin catabolism However, it

[4] *Sadtler Standard Infra-red Spectra* Sadtler Research Laboratories Inc , Philadelphia, Penn (1969)
[5] W A BOUGH and J E GANDER, *Phytochem* **10**, 67 (1971)

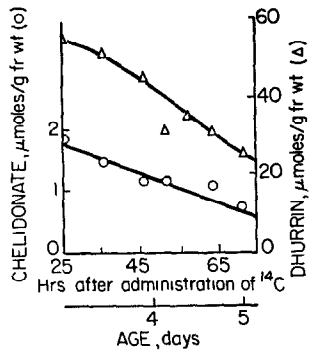

FIG. 1 CHANGE IN THE AMOUNT OF DHURRIN AND CHELIDONIC ACID WITH AGE OF SEEDLING

Two-day-old etiolated sorghum seedlings were held at 22° with their roots submerged in 2·5 mM L-tyrosine for 2 days. Seedlings were removed at intervals and the quantity of dhurrin and chelidonic acid in the shoots estimated by procedures described in the Experimental, or described previously [5]. Chelidonate was estimated by its absorbance at 270 nm following its separation from dhurrin by chromatography on PVP and its separation from tyrosine by extraction into acidic ether.

was of interest to determine if exogenous L-tyrosine could serve as a precursor of chelidonic acid.

Etiolated sorghum seedlings were allowed to metabolize exogenously supplied L-tyrosine labelled with ^{14}C. Chelidonic acid was separated from dhurrin by chromatography on PVP. The fractions containing tyrosine and chelidonate were concentrated and chromatographed on paper in BuOH–pyridine–H_2O (6 . 4 : 3, v/v). Tyrosine (R_f 0·40) separates from chelidonic acid (R_f 0·10) and both areas of the chromatogram were radioactive. The chelidonate was eluted and the specific activity estimated in both dhurrin and chelidonate.

TABLE 1 INCORPORATION OF ^{14}C FROM L-TYROSINE INTO DHURRIN AND INTO CHELIDONIC ACID

Position of label in L-tyrosine	Specific activity		Dilution in specific activity*	
	Dhurrin–Chelidonate		Dhurrin	Chelidonate
	(dis/min/μmol)			
C-2	1024	180	500	2900
C-3	830	435	450	860
All C atoms	1200	804	375	560

Three-day-old etiolated seedlings were allowed to take up and metabolize 10 μc [^{14}C]L-tyrosine from 50 ml solution. The seedlings were held at 22° in the dark for 4 days. The shoots were then excised and extracted with 0·3 M $HClO_4$. Following neutralization with KOH and removal of insoluble $KClO_4$, dhurrin and chelidonate were separated on PVP and the chelidonate separated from other phenolics by extraction into ether followed by chromatography on paper. The quantity of the two substances was estimated spectrophotometrically and the radioactivity estimated with a scintillation spectrometer.

* The dilution in specific activity is the ratio of initial specific activity of [^{14}C]L-tyrosine in the nutrient solution divided by the specific activity of dhurrin or chelidonate isolated.

Table 1 shows the specific activity of chelidonate was 2- to 6-fold less than dhurrin isolated from the same seedlings. However, the dilution factor is too great to allow a definite conclusion as to the importance of tyrosine as a precursor of chelidonate We have shown previously[5] that in these kinds of experiments the dilution of dhurrin results from at least two factors operating together, (1) 95%, or more, is derived from endogenous precursors, and (2) the relatively large pool of dhurrin Although the data do not exclude the possibility that dhurrin or one of its metabolic degradation products might be a precursor in chelidonate biosynthesis, they clearly show that exogenous L-tyrosine is not an effective precursor of chelidonic acid

Bohm[6] showed that either glucose or ribose served as precursors of chelidonate in flowering *Convallaria majalis* In these experiments isotope dilution values of 720, 940 and 11,000 were reported for glucose, ribose and acetate respectively He also reported that [^{14}C]shikimate was not incorporated into chelidonate

The role of chelidonate in *Sorghum vulgare* or any other plant is unknown Leopold *et al*[7] showed that 10 μM chelidonate inhibited plant growth, but that in the presence of indole acetic acid it stimulated elongation of *Avena* coleoptiles A pK_a of about 2·4 for both carboxyl groups[8] makes it a very effective chelating agent at physiological pH The physiological role of chelidonate in young plants may relate to this property

EXPERIMENTAL

Reagents The [2-^{14}C]- and [U-^{14}C]-L-tyrosine were purchased from Calbiochem and [3-^{14}C]-DL-tyrosine was purchased from New England Nuclear Corporation L-Tyrosine was isolated from DL-tyrosine as described previously[5] Insoluble polyvinylpyrrolidone (PVP) was purchased from General Aniline and Film Corporation Dowex-1-X8 resin was purchased from Calbiochem as an analytical grade reagent All other chemicals used were reagent grade

Germination and growth conditions Seeds of *Sorghum vulgare* cv Rox Orange were a gift of Northrup, King and Company or were obtained from Foundation Seed Division, College of Agriculture, Lincoln, Nebraska The seeds obtained from Nebraska had been treated with Captan 75-Malathion 1 5 seed protectant, and the seeds were washed thoroughly to remove as much of the pesticide as feasible The seedlings were germinated and grown in the dark at 22° in glass trays containing moist vermiculite All operations which required manipulations of the seedlings or support medium were performed under dim green light

Extraction of seedlings for isolation of chelidonic acid The shoots were separated from the roots and seeds at the indicated time after planting them in vermiculite, usually between the fifth to ninth day The shoots were extracted with 3 vol of cold 0 3 M perchloric acid per g fresh wt of tissue When the fresh wt was 10 g or less, the tissue was macerated with a chilled mortar and pestle using sand as an abrasive A Waring blendor was used to homogenize larger amounts of tissue The homogenate was extruded through cheese cloth to remove fibrous material and the filtrate adjusted to pH 5–6 with 10 N KOH The extract was centrifuged at 18,000 *g* for 15 min to remove the insoluble K-perchlorate The supernatant solution was then passed onto the appropriate column of Dowex-1-bromide or chloride All operations were carried out at 4° when feasible

Chromatography on Dowex-1-bromide A 1 5 × 40-cm column of washed Dowex-1-bromide resin was charged with an extract of sorghum prepared as described above The column, charged with an extract from 10 to 50 g fresh wt of shoots, was irrigated with a solution containing KBr the concentration being regulated by a gradient maker apparatus Fractions having a vol of 15 ml were collected at a flow rate of 30–50 ml/hr Chart paper was driven at a rate of 1 cm/hr and the transmission at 254 nm recorded continuously

Chromatography on PVP A 1 5 × 40-cm column of purified PVP was charged with 2 ml of a pH 3–4 extract obtained from 0 1 to 10 g fresh wt of sorghum shoots The column was irrigated with deionized distilled water at a rate of approximately 10 ml/hr Five ml fractions were collected and the percentage transmission recorded continuously Reay[9] also used PVP for dhurrin isolation

Paper chromatography Samples were applied to Whatman No 3 MM paper and run in *n*-BuOH–pyridine–H_2O (6 4 3, v/v) by ascent Chromatography was also conducted on Whatman No 1 paper using benzene–

[6] B A BOHM, *Arch Biochem Biophys* **115**, 181 (1966)
[7] A LEOPOLD, F I SCOTT, W H KLEIN and E RAMSTAD, *Physiol Plant* **5**, 85 (1952)
[8] S MIYAMOTO and E BROCHMANN-HAMSSEN, *J Pharm Sci* **51**, 552 (1962)
[9] P F REAY, *Phytochem* **8**, 2259 (1969)

HOAc–H_2O (125 72 3, v/v)[10] The dried chromatograms were viewed under UV light (253 7 nm) and the fluorescent or fluorescence quenched spots outlined

Elemental and qualitative analyses Elemental analyses was performed by Huffman Laboratories, Inc , Wheatridge Colorado , Mol wt was estimated by an ebulliscopic method and by potentiometric titration in 30% MeOH

Dhurrin and chelidonic acid specific activity from [^{14}C]-L-*tyrosine* Three-day-old etiolated seedlings were placed in a solution containing 10 μc L-tyrosine labeled with ^{14}C in the indicated positions The seedlings were held at 22° for 4 days and the shoots harvested and extracted with 0 3 M perchloric acid as described above Chelidonic acid was separated from dhurrin by chromatography of the extract on PVP followed by extracting the fractions containing chelidonic acid into ether The quantity of chelidonic acid was estimated by measuring its absorbance at 270 nm and converting this to μmoles based on a molar extinction coefficient of 1 1 $\times$ 10^4 The radioactive sample was chromatographed in either the BuOH–pyridine–H_2O or benzene–HOAc–H_2O to separate chelidonic acid from tyrosine and the distribution of radioactivity determined with a Baird-Atomic radiochromatogram strip scanner The quantity of dhurrin in the fractions eluted from the PVP was estimated spectrophotometrically as described previously [5] The radioactivity in the samples was estimated in a scintillation spectrometer and the counts corrected to give disintegrations per minute

Large scale isolation and purification of chelidonic acid 9-day-old etiolated shoots (1590 g) were extracted with 3 vol of 0 3 M perchlorate by homogenization in a Waring blendor After filtration through cheese cloth, the filtrate was adjusted to pH 5 2 with 10 N KOH and filtered The filtrate was further clarified by centrifugation The filtrate was applied to a column of Dowex-1-Cl, bed vol 550 ml The column was washed with water followed by a continuously increasing concentration of KCl The KCl gradient was generated in the following system mixing flask, 500 ml H_2O, main reservoir charged successively with 1000 ml 1 M KCl, 500 ml 2 M KCl, 650 ml 3 M KCl and 500 ml 3 M KCl in 1 N HCl The chelidonic acid eluted in a vol of 435 ml after 2265 ml of the KCl gradient had passed through the column The 435 ml contained 17,820 absorbance units at 270 nm The 435 ml were neutralized and concentrated to 180 ml *in vacuo* at 35° The solution was then extracted continuously with ether for 2 days The ether extract was discarded and the aqueous phase acidified to pH 1 and the solution extracted with ether continuously with ether for 7 days The ether was evaporated *in vacuo* at 20°, or less, and the oil that remained was dissolved in 10 ml H_2O This solution contained 16,190 absorbance units when measured at 270 nm and was applied to a 4 5 $\times$ 37 cm column of PVP The column was developed with 3300 ml H_2O followed in turn by 500 ml 1 N HCl, then 500 ml 0 5 M KCl in 1 N HCl and finally 300 ml 1 M KCl in 1 N HCl Chelidonic acid was contained in the fractions eluting between 2115 and 4305 ml, and these combined fractions contained 14,080 absorbance units when measured at 270 nm The volume of the combined fractions was reduced *in vacuo* at 35° to 180 ml The solution was adjusted to pH 1 and extracted with ether continuously for 6 days 10 ml H_2O was added to the ether extract and the ether evaporated *in vacuo* at 20° or less A white precipitate formed during evaporation of the ether The solution was warmed gently and a minimum volume of warm water added to dissolve the precipitate Needle-shaped crystals were formed upon standing at 4°

Spectral and chromatographic analyses indicated that additional purification was needed The crystals were dissolved in water, the solution adjusted to pH 6 and rechromatographed on PVP in water The fractions containing chelidonic acid were extracted into acidic ether as described previously, and the ether dried 10 ml H_2O was added to the ether and the ether removed *in vacuo* at 20° or less The solution was then passed over a 4 5 $\times$ 40 cm Sephadex G-10 column (bed vol 700 ml) The column was irrigated with water and the fractions containing chelidonic acid were combined, the volume reduced by evaporation *in vacuo* and adjusted to pH 1 The solution was extracted with ether for 2 days 10 ml H_2O was added and the ether evaporated The residue was crystallized twice from water Approximately 7500 absorbance units when measured at 270 nm were obtained at the step prior to crystallization Of this quantity, 74 5 mg of crystalline chelidonic acid contained 4060 absorbance units when measured at 270 nm, a value of 54 5 units/mg

[10] W. Steck and S H Wender, *J Chromatog* **19**, 564 (1965)

Key Word Index—Sorghum vulgare, Gramineae, chelidonic acid, biosynthesis