Pergamon

0031-9422(93)00283-9

Phytochemistry, Vol. 40, No. 3, pp. 619–628, 1995
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0031-9422/95 $9.50 + 0.00

THE CHRYSOPINE FAMILY OF AMADORI-TYPE CROWN GALL OPINES

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(*Received* 7 *February* 1995)

Key Word Index—Crown gall; *Agrobacterium tumefaciens*; opines; Amadori reaction; chrysopine; N^2-(1′-deoxy-D-fructopyranos-1′-yl)-L-glutamine-δ-lactone; N^2-(1′-deoxy-D-fructos-1′-yl)-L-glutamine; *N*-(1′-deoxy-D-fructos-1′-yl)-5-oxo-L-proline.

Abstract—Crown gall tumours induced by four groups of *Agrobacterium tumefaciens* isolates, derived from galls from four different locations in North and South America and Europe, were found to contain mannityl opine-related metabolites derived from condensation of glucose with glutamine followed by Amadori rearrangement. The opines were characterized spectroscopically as *N*-(1′-deoxy-D-fructos-1′-yl)-5-oxo-L-proline, N^2-(1′-deoxy-D-fructos-1′-yl)-L-glutamine and its spiropyranosyl lactone, chrysopine. Tumours induced by *A. tumefaciens* strain 2788 and by *Ficus* strains also contained nopaline, while those induced by *A. tumefaciens* K224, K289 and Chry9 contained L, and an unidentified opine, pseudo-nopaline.

INTRODUCTION

Crown gall and hairy root disease are plant diseases induced by the soil bacterium *Agrobacterium* containing either a tumour-inducing Ti-plasmid or a root-inducing Ri-plasmid [1–3]. A portion of the DNA (T-region) of the Ri-or Ti-plasmid is transferred from the bacterial plasmid into the host plant cell where the transferred DNA (T-DNA) is stably integrated into the host plant chromosome. T-DNA genes encoding production of plant hormones and plant growth regulators are expressed in transformed plant cells where they cause uncontrolled cell division leading to a tumour or to wild growth of hairy roots at the site of infection. In addition to genes encoding plant hormone biosynthesis, T-DNA contains genes encoding enzymes which modify one or more primary metabolites into secondary metabolites not normally found in the host plant. These modified metabolites, called opines are not catabolizable by the host plant [4]. However, genes for catabolism of opines are contained on the non-transferred portion of the Ti- or Ri-plasmid. These genes of opine catabolism are expressed in the bacterium in the presence of the opine, allowing the bacterium containing the Ti-(or Ri-) plasmid to grow on the opine. The manufacturing plant derives no benefit from the opine. There is a correspondence between the type of opine produced in the tumour and the type of opine catabolizable by the inducing bacterium. Production of opines in tumour cells is therefore a method by which photosynthate and nitrogen of the host plant is tied up in a form unusable by the plant, but beneficial to the tumour-inciting bacterium.

Many distinguishable Ti- and Ri-plasmids have been recognized based on structural differences in the opines synthesized in the tumour which they induce [4]. Except for the agrocinopines (arabinosylphosphosucrose and derivatives), all of the remaining opines are derived from amino acids and thus contain nitrogen. The nitrogenous opines all arise from condensation of a carbonyl compound of primary metabolism (glucose, pyruvic acid or α-ketoglutaric acid) with an L-amino acid. The resulting imine is often stabilized by reduction. Stereospecific reduction of the imine produced by condensation of pyruvic acid or glutamic acid with an L-amino acid yields either a *threo*- or *erythro*-iminodiacid [5–7]. Tumours induced by an *A. rhizogenes* strain produce cucumopine, a conjugate of α-ketoglutaric acid and histidine in which the imine is not reduced, but rather, undergoes Pictet–Spengler type cyclization with the imidazole ring to give a chiral imidazolopiperidine [8]. Cucumopine is also present in tumours incited by grapevine *Agrobacterium* strains. A different *A. rhizogenes* strain is known to effect this cyclization with the opposite stereochemistry at the new asymmetric centre producing mikimopine [9].

The mannityl opines are *N*-mannityl derivatives of glutamic acid. Mannopine (**1a**) is N^2-(1′-deoxy-D-man-

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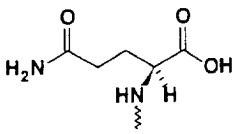

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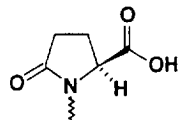

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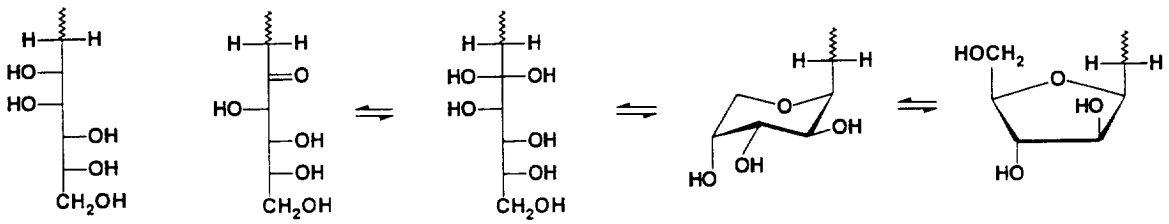
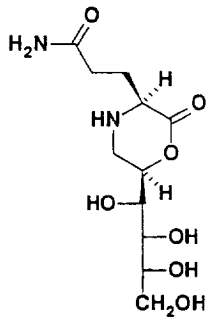


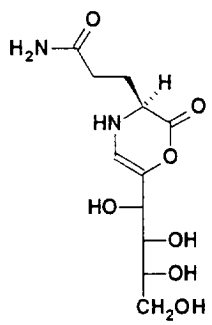
a b c d e

1a: Mannopine **1b,c,d,e: Deoxyfructosylglutamine**

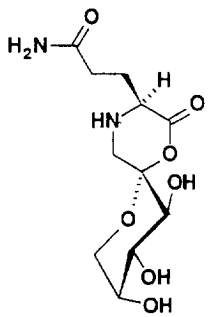
2a: Agropinic acid **2b,c,d,e: Deoxyfructosyloxoproline**



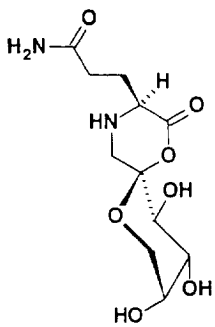
3: Agropine



4: Agropin-1-ene



5: Chrysopine β-anomer



6: Chrysopine α-anomer

nit-1-yl)-L-glutamine, mannopinic acid is *N*-(1′-deoxy-D-mannit-1′-yl)-L-glutamic acid, and agropinic acid (**2a**) is the derived lactam *N*-(1′-deoxy-D-mannit-1′-yl)-5-oxo-L-proline. Some, but not all mannityl opine overgrowths also contain agropine (**3**) the δ-lactone of mannopine [10]. A pathway of biosynthesis of mannityl opines has been proposed, and the potential intermediates have been isolated [11]. In the proposed pathway mannityl opines are derived from glucose, not mannose. Stereochemistry at C-2 of glucose is lost by Amadori rearrangement of *N*-glucosylglutamine into N^{α}-(1′-deoxy-D-fructos-1′-yl)-L-glutamine (**1b**) prior to reduction. The postulated deoxyfructosylglutamine, and the corresponding lactam, *N*-(1′-deoxy-D-fructos-1′-yl)-5-oxo-L-proline (**2b**) have been detected electrophoretically as low level intermediates in tumours which synthesize mannityl opines [11]. A further low level intermediate was detected electrophoretically in mannityl opine tumors and formulated as the unsaturated lactone agropin-1′-ene (**4**). The latter opine, along with **1b** and **2b**, was found at higher level in tumours induced by the atypical nopaline-type strain IIBV7.

The anomeric hydroxyl at C-2 of the deoxyfructosyl moiety was omitted in structure **1d**, **1e**, **2d** and **2e**.

RESULTS AND DISCUSSION

In the course of characterizing opines in tumours induced by new *Agrobacterium tumefaciens* strains we have observed in four separate collections of strains [12–16] (Table 1) the presence of a set of three tumour related products, chrysopine (**5** or **6**), deoxyfructosylglutamine

Table 1. Chrysopine-type strains of *Agrobacterium tumefaciens*

Strain	Opines catabolized*	Opines in tumour	Host plant and location	References	
				Strain isolation	Opines
K224, K289	LL-SAP, LL-LOP, CHR, dfg, dfop ψ-NOP	LL-LOP, CHR, dfg, dfop, ψ-NOP	Chrysanthemum, Colombia	L. W. Moore (unpublished)	This work,
Chry1, 3, 5, 8, 9	LL-SAP, LL-LOP, CHR, dfg, dfop, ψ-NOP	IDA, CHR, dfg, dfop, ψ-NOP,	Chrysanthemum, Florida	Ref. [12]	This work, Ref. [13]
Ficus strains					
AF3.10, 3.44 3.53, 3.81, 3.9	NOP, CHR,	NOP, CHR, dfg, dfop	*Ficus benjamina* Florida	Ref. [14]	This work
ANT4	LL-SAP, LL-LOP, CHR, dfg, dfop	CHR, dfg, dfop, LL-SAP	Chrysanthemum France	Ref. [15]	This work
2788	NOP, CHR, dfg, dfop	NOP, CHR, dfg, dfop	Chrysanthemum France	Ref. [15]	This work
IIBV7	NOP, other not tested	NOP, dfg, dfop, 'agropin-1-ene'	Chrysanthemum, Germany	A.C. Braun cited in Ref. [16]	Ref. [11]

*Abbreviations: CHR, chrysopine; dfg, deoxyfructosylglutamine; dfop, deoxyfructosyloxoproline; LL-LOP, L,L-leucinopine; L,L-SAP, L,L-succinamopine; IDA unspecified iminodiacid (could be LL-LOP or LL-SAP); ψ-NOP, pseudonopaline.

(**1b**) and deoxyfructosyloxoproline (**2b**), having similar electrophoretic properties and silver nitrate staining as the mannityl opines, agropine (**3**), mannopine (**1a**) and agropinic acid (**2a**), respectively [15]. Although these tumour-related metabolites have the same electrophoretic mobilities as the mannityl opines at pH 1.8–6.0 (Table 2), they are readily distinguishable from the mannityl opines by their reduction of colourless triphenyltetrazolium chloride to red triphenylformazan at room temperature. Mannityl opines do not react with this reagent at room temperature. The FAB-MS of the three new opines showed that each had M_r two mass units lower than that of the mannityl opine with which it co-migrated when electrophoresed at pH 1.8. This, coupled with the fact that the new opines are oxidized by triphenyltetrazolium chloride, suggested that the new opines are the deoxyfructosyl analogues of the mannityl opines derived by Amadori rearrangement of *N*-glucosyl-L-glutamine. These Amadori opines are referred to by chemical names used by Ellis *et al.* [11], except for the agropine analogue chrysopine, which has a structure isomeric to that assigned in the previous work.

The mannopine-like opine N^2-(1′-*deoxy*-D-*fructos*-1′-*yl*)-L-*glutamine* (**1b**)

An *A. tumefaciens* strain which does not catabolize deoxyfructosyl-glutamine was inoculated to a tumour extract to deplete the primary amino acids and other opines. Deoxyfructosylglutamine in the biologically depleted extract was purified by preparative electrophoresis at pH 1.8. Deoxyfructosylglutamine was noticeably less stable in purified preparations than in crude plant extracts at pH > 5. However, slight residual acidity in preparations recovered from electrophoresis at pH 1.8 rendered solutions of the opine considerably more stable. The opine was stored at $-20°$ after evaporation of water. Synthetic N^2-(1′-deoxyfructos-1′-yl)-L-glutamine and the new opine co-migrated on electrophoresis in the pH range 1.8–10 and co-migrated on paper chromatography developed with butanol–acetic acid–water (12:3:5). Deoxyfructosylglutamine co-migrates with mannopine on electrophoresis over the range 1.8–6.0, but is more mobile and well-resolved from mannopine at pH 8.0 (Table 2). At pH 10.0 the mobility of mannopine is almost as great as that of deoxyfructosylglutamine. In the range pH 8–10 mobility of these two opines is affected by progressive deprotonation of the secondary ammonium group. The electrophoretic mobility data indicate that the amino group of deoxyfructosyl glutamine is about one order of magnitude less basic than the amino group of mannopine. Deoxyfructosylglutamine is a weaker base than mannopine because of the inductive effect of two β-oxygens in deoxyfructosylglutamine while mannopine has only one β-oxygen.

Opine obtained from the tumour had ^1H and ^{13}C NMR spectra indistinguishable from spectra of synthetic deoxyfructosylglutamine (Table 3). Carbon signals of minor anomers were evident in both the natural and synthetic opine. Analysis of ^{13}C NMR of a large number of *N*-(1′-deoxyfructos-1′-yl)-amino acids has shown that the dominant anomer is the β-pyranose ($^2C_5$ chair conformation) with small, but detectable amounts of α- and β-furanose as well as α-pyranose [17].

Catabolic studies were used as a further test of the identity of synthetic deoxyfructosylglutamine and the new opine. However, catabolic tests lasting more than three or four days were difficult to conduct in the defined AT medium [18] because of the oxidative instability of

Table 2. Electrophoretic mobilities of Amadori opines and mannityl opines

Compound	pH 1.8*	2.8†	4.0‡	6.0§	8.0¶	10.0‖
Chrysopine	0.37	0.04	0	0	0	0
Agropine	0.37	0.04	0	0	0	0
Deoxyfructosylglutamine	0.26	0.02	0	0	−0.51	−0.70
Mannopine	0.26	0.02	0	0	−0.21	−0.63
Deoxyfructosyloxoproline	0	−0.33	−0.69	−0.82	−0.80	−0.80
Agropinic acid	0	−0.33	−0.69	−0.82	−0.80	−0.80

*pH 1.8 = formic-acetic acid; mobility relative to 2-[*p*-(dimethylamino)styryl]-1-methylpyridinium iodide at pH 1.8.

†pH 2.8 = 0.1 M formic acid; mobility relative to picric acid at pH 2.8–1.0. Negative sign preceding mobility indicates movement in anionic direction.

‡pH 4.0 = 0.1 M acetic acid.

§pH 6.0 = 0.5 M pyridine.

¶pH 8 = 0.1 M morpholine buffer.

‖pH 10 = 0.1 M ammonia buffer.

Table 3. NMR comparison of natural and synthetic deoxyfructosylglutamine

H, C #	^{1}H NMR Synthetic	^{1}H NMR Natural	^{13}C NMR Synthetic	^{13}C NMR Natural
Glutaminyl moiety				
α	*ca* 4.8	*ca* 4.8	*	*
βa,b	2.13 *m*	2.02 *m*	24.50	26.09
γa,b	2.47 *m*	2.39 *m*	30.69	30.12
α-CO			177.39	Not observed
γ-$CONH_2$			172.62	Not observed
Deoxyfructosyl moiety				
1a	3.29	3.15 *d*, *J* = 13 Hz	52.18	53.09
1b		3.23 *d*, *J* = 13 Hz		
2 (ketal)			94.96	95.5
3	3.71 *m*	3.79 *dd*, *J* = 3, 10 Hz	68.47	69.21 |
4	3.85 *m*	3.61 *m* |	68.08	69.7 |
5	3.97 *m*	3.67 *m* | arbitrary	69.47	70.33 |
6a	3.71 *m*	3.56 *m* | assign.	63.46	64.33
6b	3.97 *m*	3.91 *m* |		

*C-α of glutaminyl moiety not assigned because water signal prevented ^{1}H–^{13}C correlation and because of presence of ^{13}C signals of equilibrating isomers.

deoxyfructosylglutamine at pH 7. Unknown components in crude tumour extract prolong the lifetime of the natural opine. Similarly, addition of half-strength nutrient broth also protects synthetic and natural deoxyfructosylglutamine and permits catabolic studies of 10 days duration or more. Catabolism in half-strength nutrient broth was monitored by electrophoresis at pH 1.8 using an uninoculated control of deoxyfructosylglutamine in half-strength nutrient broth. Catabolism of the opine was judged to be complete if the opine disappeared from the inoculated sample but not from the uninoculated control. Using half-strength nutrient broth it was shown that those strains capable of catabolizing the natural opine could also catabolize synthetic deoxyfructosylglutamine within two days, while those strains which did not catabolize the natural opine within ten days failed to catabolize synthetic deoxyfructosylglutamine (data not shown).

To test the stereospecificity of catabolism, the C-4 epimer of deoxyfructosylglutamine, N^2-(1′-deoxy-D-*lyxo*-hexulos-1′-yl)-L-glutamine was prepared from D-galactose and L-glutamine. In an experiment in which deoxyfructosylglutamine was catabolized within two days by *A. tumefaciens* strains Chry9, K289, K224 and C58, the C-4 epimer was not catabolized by any of these strains within 10 days. Therefore the new opine is not derived

from D-galactose. The remaining aldohexoses are quite rare and are unlikely to be incorporated into an opine capable of being produced in whatever plant *Agrobacterium* happens to transform. In a subsequent experiment the four test strains were grown first on deoxyfructosylglutamine. The C-4 epimer was added after disappearance of the opine from the culture medium. Under these conditions the C-4 epimer disappeared from the medium in less than 24 hr. Apparently, the transport and catabolism enzymes are induced by deoxyfructosylglutamine, but not by the C-4 epimer. However once the catabolic and transport enzymes are induced they are capable of efficient and complete uptake and catabolism of *N*-(1′-deoxy-D-*lyxo*-hexulos-1′-yl)-L-glutamine. This suggests that, as with mannityl opine strains of *Agrobacterium*, there is greater stereospecificity in inducer recognition than in transport and catabolism of the opine [19].

Relative acid–base stability of deoxyfructosylglutamine and chrysopine

Chrysopine, mannopine and agropine are much more stable than deoxyfructosylglutamine. The relative stabilities of sterile solutions of chrysopine and deoxyfructosylglutamine (both at 15 mg ml^{-1}) were compared at room temperature in 0.1 M HCl, in 0.01 M sodium dihydrogen phosphate (pH 4.25), and in 0.01 M sodium monohydrogen phosphate (pH 8.9). Intensities of silver nitrate-developed spots were estimated following paper electrophoresis at pH 1.8. Deoxyfructosylglutamine was nearly completely destroyed within three days both in 0.1 M HCl and at pH 8.9. There was little loss of deoxyfructosylglutamine at pH 4.25 within six days, but this opine did disappear from the sterile, pH 4.25 solution by 20 days. In a separate experiment the decomposition rate of deoxyfructosylglutamine increased progressively as the sodium phosphate buffer was made more alkaline from pH 4.25–5.85, 6.90 and 8.70. The maximum stability occurs somewhere in the pH range 1–4. The lifetime of deoxyfructosylglutamine at pH 6.90 was greatly increased by addition of 4 mg of Difco nutrient broth per millilitre. Amadori compounds are known to undergo facile oxidation at room temperature in alkaline solution liberating the amine, an osone and oxidative cleavage products of the sugar [20]. The oxidation to glucosone is catalysed by trace copper contamination in water. Nutrient broth and crude tumour extract may protect deoxyfructosylglutamine either by providing an alternative sacrificial reductant or by chelating traces of copper.

Deoxyfructosylglutamine is lactamized to deoxyfructosyloxoproline in 0.1 M HCl. Degradation of deoxyfructosylglutamine at pH 4.25 slowly produced deoxyfructosyloxoproline, glutamine and a neutral tetrazolium-positive material. Glutamine and neutral tetrazolium-positive material, but no deoxyfructosyloxoproline, were produced at pH 8.9. The neutral tetrazolium-positive material reacted on the electrophoretogram with phenylhydrazine at room temperature to give a yellow product, presumably phenyl glucosazone formed from glucosone. Chrysopine was much more stable than deoxyfructosylglutamine under all conditions. It underwent the least degradation in 0.1 M HCl. Some loss of chrysopine was detectable after 20 days at pH 4.25. More extensive, but not complete loss occurred at pH 8.9 after 20 days at room temperature.

The agropinic acid-like opine N-(1′-*deoxy*-D-*fructos*-1′-*yl*)-5-*oxo*-L-*proline*

The opine co-migrating with agropinic acid was enriched by batch adsorption to and elution from an anion exchange resin followed by passage through a cation exchanger. The enriched opine was then purified by preparative electrophoresis

Synthetic *N*-(1′-deoxy-D-fructos-1′-yl)-L-glutamic acid was prepared from D-glucose and L-glutamic acid via Amadori rearrangement. The deoxyfructosylglutamic acid was absorbed onto an acidic cation exchange resin and lactamized by heating to give the known compound *N*-(1′-deoxy-D-fructos-1′-yl)-5-oxo-L-proline [17]. Sythetic deoxyfructosyloxoproline and natural opine comigrated on electrophoresis in the pH range 2.8–10 and were immobile at pH 1.8. Both the synthetic and natural opine gave the quasimolecular $[M - H]^-$ ion at *m/z* 290 and an oxoprolyl fragment at *m/z* 128 ($C_5H_6NO_3$) in the negative ion FAB-mass spectra. The ^1H and ^{13}C NMR of the synthetic and natural opines were indistinguishable (data not shown) and in agreement with the spectra reported by others for this compound [17, 21]. A weaker set of proton signals of a second anomer was present in both the synthetic and tumour-derived deoxyfructosyloxoproline. The major isomer (*ca* 80%) has been described as the β-pyranose and the minor isomer as α-furanose (*ca* 15%) [21].

Both synthetic deoxyfructosyloxoproline and the natural opine were catabolized by chrysopine strains Chry9, K224 and K289. In addition, both were catabolized by the agropine/leucinopine strain Bo542. Neither were catabolized by nopaline strain C58 nor by *Ficus* strains (AF3.10 and 3.44). These observations are consistent with more extensive catabolic experiments on the natural opine presented in a separate communication [15].

Chrysopine, the opine comigrating with agropine

Small batches of chrysopine were isolated by ion exchange and preparative electrophoresis from a tobacco tumour line induced by strain Chry9, from sunflower tumour induced by strain K289, and from tomato tumour induced by *Ficus* strains. The identity of the opine from these sources was verified by co-electrophoresis at pH 1.8 and by demonstrating that the inducing strains could catabolize the opine produced by the other two chrysopine-type strains. Chrysopine was isolated preparatively from Chry9-induced tobacco tumour by absorption onto cation exchange resin and elution with dilute ammonia. Amino acids in the crude chrysopine fraction were removed by inoculating this fraction with *A. tumefaciens* Bo542, a strain which catabolizes common amino acids, agrocinopine C and D, and leucinopine, but does not catabolize chrysopine [15]. Following removal

of bacteria by centrifugation, chrysopine was absorbed onto a cation exchange resin and eluted with dilute ammonia. The chrysopine was further purified by preparative electrophoresis followed by Sephadex G-10 chromatography. Electrophoretograms of purified chrysopine were free of impurities detectable with ninhydrin, silver nitrate or tetrazolium reagents. The isolation yield of chrysopine, 0.4 mg g^{-1} fresh tumour tissue, represents a lower limit on the amount of chrysopine present in tumours.

Chrysopine co-migrates with agropine as a cation at pH 1.8, and is, like agropine, electrophoretically immobile between pH 2.8 and 10. The unusually weak basicity of the agropine amino group has been ascribed to the base-weakening effect of a β-acyloxy group (lactone) and to hydrogen bonding between the carboxamide and the cyclic amino group [10]. The same structural features may be responsible for the very weak basicity of the amine in chrysopine. On electrophoresis of chrysopine in 0.02 M NaOH (pH 11.4) a single anion was detected with both silver nitrate and tetrazolium reagents. The anion had the electrophoretic mobility of deoxyfructosylglutamine which is probably produced by rapid hydrolysis of the lactone portion of chrysopine. Deoxyfructosylglutamine is extremely unstable under alkaline conditions. As expected, if the electrophoresis of chrysopine is delayed 30 min after wetting with 0.02 M NaOH, no anionic substance is detectable with either the silver nitrate or tetrazolium reagents.

The FAB mass spectra of purified chrysopine possessed a quasimolecular ion at m/z 291 in positive ion mode and m/z 289 in negative ion mode, indicating a M_r of 290, two mass units lower than that of agropine. The M_r of chrysopine and its co-electrophoresis with the lactone agropine in the pH range 1.8–10 are compatible with the presence of a lactone in chrysopine. The ^{13}C NMR spectrum of chrysopine (Table 4) shows the presence of a ketal carbon at 98.7 ppm. Thus chrysopine is a pyranosyl (or furanosyl) ketal.

The large coupling between H-3 and H-4 (J_{34} = 10 Hz) would be expected of a pyranosyl derivative in the chair conformation. Chrysopine is electrophoretically immobile at pH 8–10, but migrates as an anion in borate buffer (pH 9.2). Model methyl-α- and β-D-fructopyranosides are also mobile in borate buffer due to borate ester formation with *cis* hydroxyls at C-4 and C-5, while methyl-α- and β-D-fructofuranosides are immobile because the *trans*-hydroxyls at C-3 and C-4 are too far apart in the five-membered ring to form a cyclic ester with borate [22]. The electrophoretic mobility of chrysopine in borate buffer is inconsistent with a furanoside but consistent with a pyranoside structure.

The NMR spectra of deoxyfructosylglutamine, deoxyfructosyloxoproline and other deoxyfructosyl Amadori compounds which are hemiketals [17, 21, 23] show the presence of a dominant β-pyranosyl isomer, along with detectable α-pyranose and α- and β-furanose. However, the spectrum of chrysopine shows the presence of a single isomer. This indicates that the lactone moiety of chrysopine involves the ketal hydroxyl, rendering chrysopine incapable of mutarotation. The oxidative instability of deoxyfructosylmannopine and other Amadori products at pH > 5 is due to oxidation of the enaminol anion. Chrysopine is much more stable than deoxyfructosylglutamine over the range pH 5–10 because the ketal hydroxyl is masked by lactonization, preventing enoliz-

Table 4. NMR spectra of chrysopine in D_2O

H, C identity	^1H NMR	^{13}C NMR	^1H–^{13}C multiple bond correlation
Glutaminyl moiety			
βa	2.14 *m*		
		25.10	2.97 (H-γa,b)
βb	2.55 *m*		
γa,b	2.97 *m*	30.45	2.55 (H-βb), 4.58 (H-α)
α	4. 58 *dd*, J = 3.5, 9.5 Hz	71.04	2.14 (H-βa), 2.55 (H-βb), 2.97 (H-γa,b)
γ-CO (amide)	--	172.3	2.97 (-γa,b)
α-CO (lactone)	--	177.6	None observed
Fructosyl moiety			
1*a*	3.65 *m**	60.80	3.65 (H-3)
1b	3.73 *m*†		
2 (ketal)	--	98.73	3.65 (H-1a,3) 3.71 (H-1b) 3.98 (H-5 or 6)
3	3.65 *m**	69.45	
4	3.86 *dd*, J = 3.2, 9.8 Hz	69.59	3.73 (H-1b or 6a), 3.98 (H-5 or H-6b)
5	3.98 *m*‡	39.83	
6a	3.73 *m*†	63.95	None observed
6b	3.98 *m*‡		

*†‡Overlapping multiplets of hydrogens on separate carbon atoms.

ation and subsequent deprotonation and oxidation. However, in 0.02 M NaOH the lactone is saponified and the deoxyfructosylglutamine released is rapidly oxidized.

A transient, tetrazolium-positive intermediate appeared during the catabolism of deoxyfructosylglutamine by *A. tumefaciens* strains Chry9 and K289. The intermediate had the same electrophoretic mobility at pH 1.8 as chrysopine, suggesting that the chrysopine strains may have an enzyme capable of interconverting deoxyfructosylglutamine and chrysopine, similar to mannopine cyclase present in agropine-utilizing strains of *A. tumefaciens* [24]. Further evidence that deoxyfructosylglutamine is convertible into chrysopine by lactonization is provided by the action of *E. coli* DH5α (pUC18PSS) on deoxyfructosylglutamine. The plasmid pUC18PSS contains the gene for the mannopine lactonizing enzyme derived from the *A. tumefaciens* plasmid pTi15955 [24]. Toluene-permeabilized cells of *E. coli* DH5α (PUC18PSS) pre-induced with mannopine not only lactonized mannopine into agropine, but also converted deoxyfructosylglutamine into chrysopine nearly quantitatively. Similarly, toluene-treated cells of chrysopine strains Chry9, K289 and ANT4 grown on deoxyfructosylglutamine were able to convert deoxyfructosylglutamine into chrysopine nearly quantitatively, but they did not convert mannopine into agropine. Chrysopine prepared by lactonization of synthetic deoxyfructosylglutamine in the presence of toluene-treated cells of *E. coli* DH5α was catabolized by the chrysopine strains Chry9, K289 and AF 3.9, but not by the nopaline strains C58. Thus, chrysopine prepared by lactonization of deoxyfructosylglutamine with mannopine cyclase has the same anomeric configuration as natural chrysopine.

The three-dimensional shape of the true substrate (**1a**) for mannopine cyclase suggests that the acyclic, hydrated form (**1c**) of deoxyfructosylglutamine may be the substrate analogue for mannopine cyclase, rather than a pyranosyl or furanosyl form. It is probable that lactonization involves the prochiral 2*R* ('manno') hydroxyl of the hydrated form rather than the prochiral 2*S* ('gluco') hydroxyl. However, knowledge of the absolute configuration of this intermediate does not allow unambiguous assignment of the anomeric configuration of chrysopine because subsequent pyranosyl ring formation must involve rupture of the C_2–OH bond at the anomeric centre. The full structure of chrysopine is either the β-D-fructopyranoside (**5**) or the α-D-fructopyranoside (**6**).

A. tumefaciens strain IIBV7 also was previously found to induce tumours producing nopaline, deoxyfructosylglutamine, deoxyfructosylglutamic acid, and a large amount of an opine detectable with tetrazolium reagent which had the electrophoretic mobility we describe for chrysopine [11]. The opine in tumours induced by strain IIBV7 was characterized by electrophoresis and chemical reduction to subtances co-migrating with mannopine and glucopine, but it was not examined spectroscopically. The opine was formulated as the enol lactone agropin-1-ene (**4**), however, it was noted that a cyclic form could not be excluded by the data [11]. We have not directly examined the opine produced by IIBV7 tumours, but it is probable that this opine is the pyranosylspirolactone, chrysopine, which contains a ketal carbon but no vinyl carbon resonance in the ^{13}C NMR.

Of the three tumour-related products only chrysopine meets the strictest definition of an opine in that it is present only in tumours induced by chrysopine strains and not in healthy plant tissue, and it is catabolized only by chrysopine strains [15]. The other two tumour-related metabolites deoxyfructosylglutamine and deoxyfructosyloxoproline, are present in tumours induced by chrysopine strains, but they are catabolized by several additional strains which do not induce tumours containing these metabolites [15]. The *Ficus* group of chrysopine strains can utilize chrysopine, but not deoxyfructosyl-glutamine even though they induce tumours producing both chrysopine and deoxyfructosylglutamine.

Chrysopine-type *A. tumefaciens* strains have been recovered from galled plants in five separate locations in South America, Europe and North America, (Table 1). It is notable that four of the five isolations were from galled chrysanthemums.

Other opines in chrysopine type tumours

All tumours induced by the four classes of chrysopine strains examined contained additional opines (Table 1). Tumours induced by *Ficus* strains of *A. tumefaciens* and by strain 2788 contained nopaline, identified by co-electrophoresis with a nopaline standard as a cation at pH 1.8 and as an anion at pH 4. The *Ficus* strains of *A. tumefaciens* and strain 2788 catabolized nopaline while *A. tumefaciens* K224, K289, ANT4 and Chry9 did not. No nopaline was detectable in tumours induced by *A. tumefaciens* K224, K289, ANT4 or Chry9. All tumours induced by K224, K289, ANT4 and Chry9 contained iminodiacid opines co-migrating with L',L-leucinopine and L',L-succinamopine. Crystalline L,L-leucinopine, having ^1H NMR identical to synthetic L,L-leucinopine was isolated from sunflower tumour induced by *A. tumefaciens* K289. Additional iminodiacid opines present in the mother liquor of crystallization of leucinopine were not identified. *Agrobacterium tumefaciens* K224, K289, ANT4 and Chry9 stereospecifically catabolized the L,L-(erythro)-isomer of a wide range of synthetic iminodiacids including L,L-succinamopine, and L,L-leucinopine. Tumours induced by strains K224, K289 and Chry9 also contained a further opine detectable with the guanidine-detecting reagents phenanthrenequinone and pentacyanoaquoferriate. This opine comigrated with nopaline and was resolvable from octopine, iso-nopaline (L,L-isomer), nopaline lactam and iso-nopaline lactam on electrophoresis at pH 1.8. However, it had zero mobility at pH 2.8–4 and was readily resolvable from the anionic nopaline and iso-nopaline at pH 4. This tumour metabolite meets the definition of an opine in that it is present in tumour tissue induced in sunflower, tobacco, tomato, and pine, but not in healthy plant tissue, it is catabolized by the inciting strains, but not by the avirulent strain A136 lacking the Ti-plasmid, nor is it catabolized by the nopaline strain A208. The new opine,

pseudo-nopaline, may easily be mistaken for nopaline on electrophoresis at pH 1.8.

EXPERIMENTAL

Spectroscopy. NMR spectra were measured on a GE Omega 500 spectrometer in D_2O using internal dimethylsilapentane sulphonate or external tetramethylsilane as reference. Proton and carbon assignments were made using DEPT, homonuclear and heteronuclear correlation spectroscopy. FAB mass spectra were measured on a JEOL HX110HF mass spectrometer using thioglycerol and 3-nitrobenzyl alcohol ionization matrices.

High-voltage paper electrophoresis (HVPE). HVPE was performed on Whatman 3 MM paper at 40 V cm^{-1}. Buffer compositions were: pH 1.8, 1 M HOAc titrated with HCOOH; pH 2.8, 0.1 M HCOOH titrated with NaOH; pH 4.0, 0.1 M HOAc titrated with NaOH; pH 8, 0.1 M morpholine titrated with HOAc, pH 10, 0.1 M NH_4OH titrated with HOAc, pH 11.4, 0.02 M NaOH. Electrophoretic mobilities of cations were measured relative to 2-(*p*-(dimethylamino) styryl)-1-methylpyridinium iodide [25] at pH 1.8, and mobility of anions was measured relative to picric acid at pH 2.8–10. The following detection reagents [4, 26] were used: 0.2% $AgNO_3$ in Me_2CO (detection of mannityl opines, Amadori opines, and leucinopine), triphenyltetrazolium chloride (selective detection of Amadori opines), phenanthrenequinone (detection of nopaline and pseudonopaline), and pentacyanoaquoferriate = sodium nitroprusside-potassium ferricyanide (detection of nopaline and pseudo-nopaline).

Biological depletion of tumour extracts. H_2O was used to extract opines from 350 g of *Datura stramonium* tumours induced by *A. tumefaciens* ANT4 [15]. Sugars, amino acids and other primary metabolites were biologically depleted by inoculation with *A. tumefaciens* ANT4 transconjugants in a C58.00 background, a plasmid-free strain which does not degrade any of the opines but does catabolize carbohydrates and amino acids present in tumor extracts. One of these transconjugants which degraded chrysopine and deoxyfructosyloxoproline but not deoxyfructosylglutamine was used to purify deoxyfructosylglutamine. The second transconjugant, which degraded chrysopine and deoxyfructosylglutamine but not deoxyfructosyloxoproline, was used to purify deoxyfructosyloxoproline. Disappearance of neutral sugars, amino acids and opines was monitored by HVPE at pH 1.8 for detection of deoxyfructosylglutamine and pH 2.8 for detection of deoxyfructosyloxoproline. Electrophoretograms were stained with $AgNO_3$ reagent and with ninhydrin. Bacteria were removed by centrifugation (10 000 *g*, 15 min, 4°), and the supernatant (250 ml) was sterilized by filtration through a 0.45 μm ultrafiltration membrane.

Isolation of deoxyfructosyloxoproline. Deoxyfructosyloxoproline and other anions contained in 100 ml of sterile, depleted extract were retained on 150 ml Dowex 1×8 OH^- in a batch absorption process. Completeness of absorption was monitored by HVPE at pH 2.8. The resin was poured into a 3×30 cm column which was washed with 2 to 3 bed vols of 10 mM NH_4OH followed by 1 vol. H_2O. Anions of weak acids were then eluted with 2 vols of 1 M HOAc. Fractions containing deoxyfructosyloxoproline were identified by HVPE at pH 2.8, pooled and concd *in vacuo* to an oily residue. The residue, dissolved in 7 ml H_2O, was percolated through a 2×30 cm column containing 50 ml Dowex 50W $\times$ $8H^+$ which did not retain the opine. Elution of the opine was completed with 2 vols of H_2O. Fractions containing deoxyfructosyloxoproline also contained a slower-migrating, Ag-chelating anion (HVPE, pH 2.8) which was removed by prep. HVPE at pH 2.8. Deoxyfructosyloxoproline (25 mg), containing a trace of the Ag chelator, was recovered from the electrophoretogram. This sample was used for spectroscopy and electrophoretic comparison to synthetic deoxyfructosyloxoproline.

Isolation of deoxyfructosylglutamine. Sterile, biologically-depleted tumour extract (100 ml) was concd at red. pres. and room temp. to 10 ml. Half of this concd soln was electrophoresed preparatively at pH 1.8. The $AgNO_3$-positive deoxyfructosylglutamine zone was eluted with H_2O. The eluate was concd to an off-white solid. Examination of this prep. of deoxyfructosylglutamine (5 mg) showed that it was free of amino acids, however it contained a small amount of $AgNO_3$ positive impurity migrating more slowly than deoxyfructosylglutamine. This sample was used for spectroscopy and electrophoretic comparison to synthetic deoxyfructosylglutamine.

Isolation of chrysopine. Tobacco tumors (800 g) induced by *A. tumefaciens* Chry9 were ground in 1.1 l of 95% EtOH. The extract was filtered through cheesecloth, and the mark was re-extracted with a further 1.9 l 80% EtOH. The combined 3 l EtOH extract was concd to 350 ml on a rotary evaporator and loaded on a 100 ml column of Dowex $50H^+$. The column was washed with 5 bed vols of Dl water. Absorbed cations were eluted with 1.5 M NH_4OH. The NH_4OH eluate was concd to an irreducible oil (2.95 g) which was dissolved in 100 ml H_2O. The soln, adjusted to pH 3, was loaded on a 70 ml Dowex $50H^+$ column. Non-retained components were eluted from the column in 220 ml Dl H_2O. The 754 mg iminodiacid fraction [5–7] was eluted in a further 1.4 l Dl H_2O. The amino acid fraction containing chrysopine was eluted with 2 M NH_4OH. Evapn gave 1.09 g non-crystalline solid.

The filter-sterilized amino acid fraction (300 mg) containing chrysopine was subjected to biochemical resolution by *A. tumefaciens* A281 in 200 ml H_2O containing 160 mg Difco nutrient broth. Catabolism of the common amino acids was complete after 48 hr on a rotary shaker at 28°. Bacteria were removed by centrifugation and the culture filtrate was loaded onto a 5 ml column of Dowex $50H^+$. The column was washed with Dl H_2O and the chrysopine (208 mg) was eluted with NH_4OH. A small amount of ninhydrin-positive material still present was removed by prep. HVPE at pH 1.8 on 6 sheets of Whatman 3 MM filter paper. The 128 mg of chrysopine re-

covered was dissolved in 1.6 ml H_2O, and 0.4 ml of this soln was loaded on a Sephadex G10 column (51 cm, 50 ml) The column was eluted with DI H_2O and 0.5 ml fractions were collected. Pooling of chrysopine-positive fractions from four column runs gave 100 mg chrysopine free of any impurities detectable by staining electrophoretograms (pH 1.8, 2.8, and 4.0) with $AgNO_3$, triphenyltetrazolium chloride, ninhydrin, and phenanthrenequinone. This prepn of chrysopine was used for spectroscopy.

Isolation of L,L-*leucinopine from tumour.* Sunflower tumours (638 g) induced by *A. tumefaciens* K289 were ground in 1 l of 95% EtOH. The extract was filtered through cheesecloth, and the mark was re-extracted with a further 0.5 l 80% EtOH. The combined 1.5 l EtOH extract was loaded on a 110 ml column of Dowex $50H^+$. The column was washed with 5 bed vols of DI H_2O. Absorbed cations were eluted with 1 M NH_4OH. The NH_4OH eluate was concd to a glass (3.9 g), re-dissolved in 20 ml H_2O and loaded on a 40 ml Dowex $50H^+$ column. Column eluants, vols and solids recovered were: DI H_2O, 100 ml, 249 mg (unretained fraction); DI H_2O, 1000 ml, 2.4 g (iminodiacid fraction); 200 ml, NH_4OH, 1.68 g (chrysopine, amino acid fraction). Electrophoresis (pH 2.8) of the iminodiacid fraction showed the presence of two cationic substances detectable as Ag-chelating white spots on a pale brown background (the major one leucinopine), as well as cations and anions oxidized to dark brown spots by $AgNO_3$. The mixture was further fractionated by elution from a 68 ml Dowex $50H^+$ column with DI H_2O. Late fractions containing a dominant iminodiacid were pooled and concd to 800 mg amorphous, white solid which required 10 ml of boiling H_2O to dissolve completely. L,L-leucinopine (133 mg) crystallized from the cooling soln and was identified by co-electrophoresis (pH 2.8) with a standard, by co-chromatography (*n*-BuOH–HOAc–H_2O, 12:3:5) and by ^1H NMR. Other Ag chelators in addition to leucinopine, presumably iminodiacids, were detectable as anions at pH 2.8 in the mother liquor of crystallization of leucinopine.

Synthesis of deoxyfructosylglutamine. Deoxyfructosylglutamine was prepared by heating glucose and glutamine in acetic acid. In order to minimize lactamization of glutamine in hot HOAc, 90 mg ml^{-1} glucose was dissolved in HOAc at 95°, then 20 mg solid glutamine was added per ml and was dissolved by stirring for 1 min. The best yields were obtained when the soln was rapidly cooled in ice H_2O 2–5 min after addition of the glutamine. Reaction time longer than 30 min completely destroyed the desired product. Longer reaction time also led to release of NH_3 by lactamization of glutamine and deoxyfructosylglutamine. The NH_3 then reacted to form β-D-glucosylamine, β,β-D-bisglucosylamine and α, β-D-bisglucosylamine which complicated the purification of the product. Deoxyfructosylglutamine was purified by prep. HVPE at pH 1.8. The order of mobility (anionic to cationic) of $AgNO_3$ detectable zones on the electrophoretogram was: (1) glutamic acid lactam and deoxyfructosyloxoproline; (2) glucose (neutral); (3) low-mobility, unknown cation; (4) deoxyfructosylglutamine; (5) β,β-D-bisglucosylamine and α, β-D-bisglucosyl-amine; (6) unreacted glutamine and glutamic acid; (7) minor unknown; and (8) β-D-glucosylamine. Deoxyfructosylglutamine was centrifugally eluted from the electrophoretogram and immediately freeze-dried and stored at −20°. The best yield of deoxyfructosylglutamine was less than 10% of glutamine used. Purity of the deoxyfructosylglutamine was checked by electrophoresis at pH 1.8 and 4.0 using ninhydrin, $AgNO_3$ and triphenyltetrazolium detection reagents.

Synthesis of deoxyfructosyloxoproline. Deoxyfructosyloxoproline was prepared via deoxyfructosyglutamic acid as described in ref [21].

Acknowledgement—The authors thank Seung Beom Hong and Stephen Farrand, University of Illinois, for providing *E. coli* DH5α (pUC18PSS), Larry W. Moore Oregon State University, for providing *A. tumefaciens* K224 and K289, and Arla Bush and Steven Pueppke, University of Missouri for providing *A. tumefaciens* Chry1, 3, 5, 8 and 9. Thanks also to Stephen Farrand for reviewing the manuscript. This research was supported in part by the North Carolina Agricultural Research Service. VVD was supported by a fellowship from the French Ministère de la Recherche et de la Technologie.

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