Carbohydrate Research, 104 (1982) 105–120
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

AGROPINE: A REVISED STRUCTURE

MAX E. TATE, JEFFREY G. ELLIS, ALLEN KERR,
Waite Agricultural Research Institute, Glen Osmond (South Australia)

JACQUES TEMPÉ,
Station de Génétique et d'Amelioration des Plantes, C.N.R.A., Versailles (France)

KEITH E. MURRAY, AND KEVIN J. SHAW
CSIRO Division of Food Research, North Ryde, N.S.W. (Australia)

(Received June 19th, 1981; accepted for publication in revised form, November 3rd, 1981)

ABSTRACT

The product known as agropine from crown-gall tumour is shown to be indistinguishable in all of its properties (physical, chemical, and biological) from N^2-(1′-deoxy-D-mannitol-1′-yl)-L-glutamine, 1,2′-lactone, a $C_{11}H_{20}N_2O_7$ compound produced by cyclisation of N^2-(1′-deoxy-D-mannitol-1′-yl)-L-glutamine. Two prior assignments of a $C_{11}H_{17}NO_7$ formula obtained by high-resolution electron-impact mass spectrometry have been shown by field-desorption mass spectrometry to correspond to a fragment produced by the rapid loss of ammonia from the parent molecular species.

INTRODUCTION

The pathogenic soil microorganism *Agrobacterium tumefaciens* induces a tumorous state in dicotyledonous plants, known as crown gall. Pathogenicity in *A. tumefaciens* has been shown to be due to the presence of a closed circular DNA molecule, the Ti plasmid, a portion of which (the T-DNA) is transferred from the bacterium to the plant cell by an as yet incompletely understood process. Apart from the morphological aspect of their unrestricted growth to form solid tumours, these genetically transformed plant cells produce "opines" characteristic of the original DNA plasmid harboured by the inducing bacteria. Most of the opines investigated so far have been found to be N^2-substituted L-amino acids, which are either absent from, or below the level of detection in, normal plant tissue. Two important features of opines are: first, that bacteria inducing their formation in plants can utilize them for their growth and, secondly, some opines promote conjugal transfer of Ti plasmids between pathogenic and non-pathogenic bacteria. These two features ensure that crown-gall tumours provide a nutritional environment that favours the pathogenic bacteria as well as aiding the dissemination of the tumour-inducing plasmid among

non-pathogenic bacteria in the vicinity of the tumour. These matters have recently been reviewed[1-3].

Agropine, an opine constituting up to 7% of the dry weight of tumours incited by strains of *A. tumefaciens* harbouring a plasmid which also induced the production of the opine known as octopine, was first reported by Firmin and Fenwick[4] as a microcrystalline product, and its molecular formula $C_{11}H_{17}NO_7$ was deduced by high-resolution electron-impact mass spectrometry. In a more detailed report, Coxon and coworkers[5] concluded that agropine has the bicyclic lactone-lactam structure **1**.

Agropine has since been isolated[3] from crown-gall tumours induced by *A. tumefaciens* strains harbouring the plasmid pTi542, and from the roots formed on plants inoculated with the related pathogen *A. rhizogenes* as well as from *in vitro* cultures derived from these roots[6].

We report here a reinterpretation of the agropine data of Coxon *et al.*[5] and propose that agropine is the 1,2′-lactone (**6**) of the reductive conjugate of L-glutamine and D-mannose. This conclusion is supported by the identity of agropine with the synthetic product (**6**) in all respects.

RESULTS AND DISCUSSION

In the present study, agropine was isolated in 0.2% fresh weight yield by a slight modification of the cation-exchange procedure of Guyon *et al.*[3] from crown-gall tumours induced on Kalanchoe by *Agrobacterium tumefaciens* strain A281 which carries the agropine plasmid pTi542.

Electrophoresis at pH 1.7 indicated the presence of minor components in the agropine fractions from the cation-exchange procedure and also in the authentic reference-samples[3,4]. At this stage, all samples were purified to homogeneity by

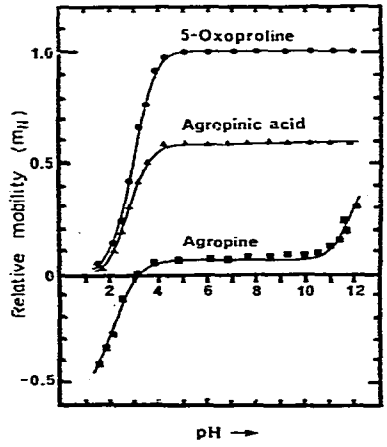


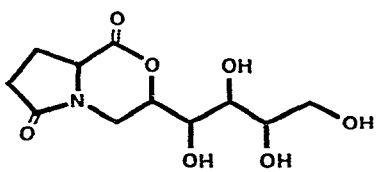
Fig. 1. pH–relative electrophoretic mobility profiles[7] for agropine, agropinic acid (**2**) and a commercial sample of 5-oxoproline (**3**). Mobilities are expressed relative to *m*-nitrobenzenesulphonate (M_N 1.0) and glycerol (m_N 0.0) as arbitrary standards of unit and zero mobility. Solid lines denote recalculated[7] mobilities by using the derived pK_a values and single-species relative mobilities in Table I.

TABLE I

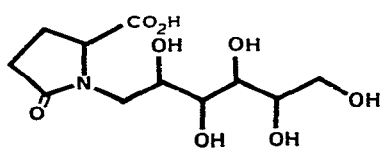
APPARENT pK_a VALUES

Compound	*Structure*	*Species net*	*Single species relative mobility* (M_N)	*Apparent*[a] *pK_a*	*Correlation coefficient*[b]
5-Oxoproline	3	0	0.0 ±0.03 (14)	2.96 ±0.03 (12)	0.95
		−1	1.02 ±0.02 (22)		
Agropinic acid	2	0	−0.02 ±0.01 (12)	2.74 ±0.02 (12)	0.98
		−1	0.58 ±0.01 (26)		
Agropine (natural)	—	+1	−0.57 ±0.04 (10)	2.15 ±0.05 (10)	0.91
		0	0.06 ±0.02 (33)	12.04 ±0.04 (12)	0.93
		−1	0.58 ±? (12)		
Agropine (synthetic)	6	+1	−0.57 ±0.04 (10)	2.15 ±0.05 (10)	0.91
		0	0.06 ±0.02 (33)	12.04 ±0.04 (12)	0.93
		−1	0.58 ±? (12)		
N^2-(1′-Deoxy-D-mannitol-1′-yl)-L-glutamic acid	4	+1	−0.47 ±? (8)	1.84 ±0.03 (8)	0.96
		0	0.07 ±0.02 (28)	3.85 ±0.03 (10)	0.97
		−1	0.56 ±0.01 (14)	9.10 ±0.04 (12)	0.93
		−2	0.98 ±0.02 (14)		
N^2-(1′-Deoxy-D-mannitol-1′-yl)-L-glutamine	5	+1	−0.47 ±0.03 (8)	1.84 ±0.03 (8)	0.96
		0	0.07 ±0.02 (20)	8.64 ±0.02 (14)	0.98
		−1	0.54 ±0.02 (28)		

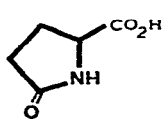
[a]Apparent ionization constants at 25° and I = 0.1–0.5 in the presence of 0.05M oxalate, and single-species relative mobilities derived[7] from the pH–relative mobility data in Figs. 1 and 5. [b]Errors and correlation coefficients obtained by least-squares solutions of linear equations[7]; values without errors were obtained by an iterative process to minimise the residual errors.



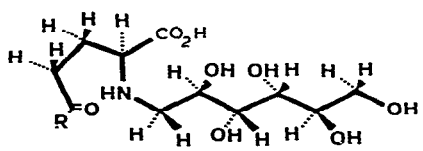
1



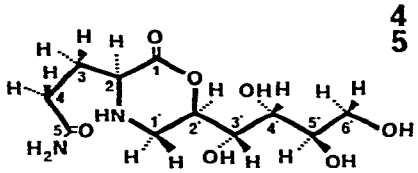
2



3



4 R = OH
5 R = NH_2



6

preparative paper electrophoresis at pH 1.7 in a volatile buffer system, before further structural and identy studies were undertaken.

As reported by Coxon *et al.*[5], alkaline degradation of agropine yields a product known as agropinic acid, for which the N^2-(1′-deoxyhexitol-1′-yl)-5-oxoproline structure (**2**) was proposed and with which we concur.

Fig. 1 shows a comparison of the pH–relative electrophoretic mobility profiles[7] for agropinic acid (**2**), 5-oxoproline (**3**), and agropine. Table I lists apparent pK_a values derived[7] from these data and also includes the relative mobilities for individual species. In the range pH 1.5–12.5, agropine behaves as a very weak base (pK_a 2.15) and a very weak acid (pK_a 12.04), whereas agropinic acid is not amphoteric and shows only the expected weak-acid ionisation (pK_a 2.74). The weak-acid properties of agropinic acid and the weak-base properties of agropine were also noted qualitatively by Coxon *et al.*[5].

An important feature shown by Fig. 1 is that no detectable protonation of the uncharged lactam species of either agropinic acid (**2**) or 5-oxoproline (**3**) occurs in the range pH 1.5–12. Hence the lactam–lactone formulation (**1**) for agropine fails to provide a satisfactory explanation as to why there should be a weak base (pK_a 2.15) as well as a very weak acid (pK_a 12.04) present in agropine and not in agropinic acid. An explanation for the cationic properties of agropine was found with the aid of field-desorption mass spectrometry (f.d.m.s.).

The presence of an ion at *m/z* 275 in the electron-impact (e.i.) mass spectrum of agropine has already been reported[4,5] and was confirmed by the present study. However, as shown in Fig. 2 under f.d.m.s. conditions, the first ion to appear in the spectrum of the purified natural product has an integer mass of 293. This ion rapidly decays as the emitter heating current is increased and is immediately followed by an ion at *m/z* 276, which represents the loss of 17 mass units. This key observation, coupled with the well documented[8–10] propensity of glutamine to undergo a facile loss of ammonia (17 mass units) to yield 5-oxoproline (**3**) prompted a simple explana-

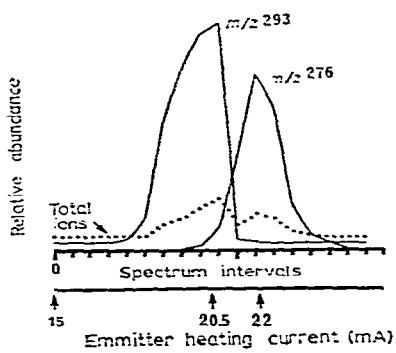


Fig. 2. Field-desorption mass-spectrometric single- (*m/z* 293 and 276) and total-ion traces as a function of the emitter heating-current. The relative abundance scales for the single-ion traces are displaced slightly from one another as well as from the total-ion trace for clarity, and the total-ion trace is compressed by a factor of 5.0 as compared with the single-ion traces.

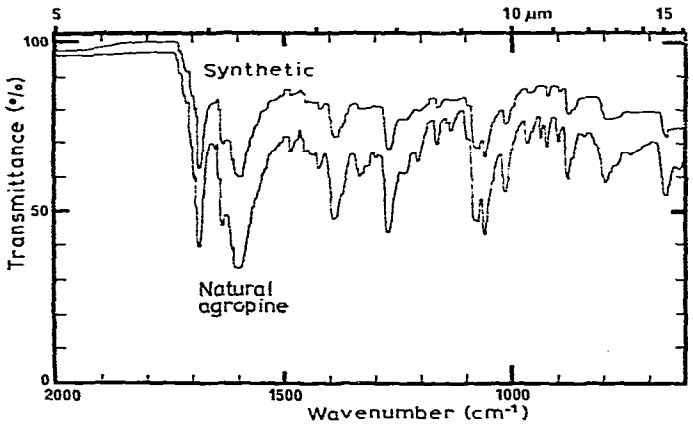


Fig. 3. Identity comparison of i.r. spectra (0.5% KCl discs) for natural and synthetic (6) agropine.

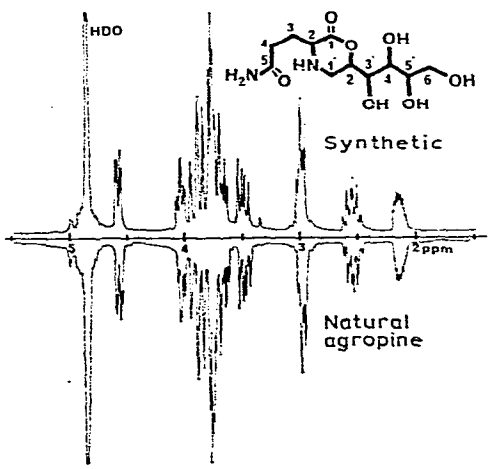


Fig. 4. Identity comparison for 270-MHz ^{1}H-n.m.r. in D_2O for natural and synthetic (6) agropine.

tion for the cationic properties of agropine shown in Fig. 1. In essence, agropine was deemed to be the 1,2′-lactone of an N^2-(1′-deoxyhexitol-1′-yl)glutamine as depicted in Fig. 4, rather than the bicyclic derivative (1) of glutamic acid proposed by Coxon *et al.*[5].

It appears that, under the conditions normally employed for e.i.m.s., lactamisation of the monocyclic $C_{11}H_{20}N_2O_7$ agropine to yield the bicyclic $C_{11}H_{17}NO_7$ structure (1) proceeds so rapidly that only the $[M - 17]^+$ ion *m/z* 275 is observed. However, in the f.d., with carefully purified agropine, the corresponding loss of ammonia from the protonated molecular ion is sufficiently slow for the individual parent $[M + H]^+$ *m/z* 293 and daughter $[(M + H) - 17]^+$ *m/z* 276 ions to be detected, as shown in Fig. 2.

The first indication of the nature of the polyol substituent was based on the

TABLE II

^{1}H-N.M.R. DATA[a]

Compound:	*Agropine (synthetic, 6)*	*Acyclic precursor (5)*	*Agropine (natural[5])*	*Agropine (synthetic, 6)*	*Agropine (synthetic, 6)*	*Agropine (synthetic, 6)*
solvent:	*Me_2SO-d_6*	*D_2O*	*D_2O*	*D_2O*	*D_2O*	*D_2O*
MHz:	*270*	*270*	*360*	*270*	*270*	*270*
Proton	*δ*	*δ*	*δ*	*δ*	*Signal*	*Coupling constants (Hz)*
H-2	4.15	3.85	4.53	4.58	dd	$^3J_{2,3A}$ 9.5; $^3J_{2,3B}$ 3.1
H-3A	2.24	2.17	2.51	2.53	m *(trans)*	$^2J_{3A,3B}$ 13.0; $^3J_{3A,2}$ 9.5; $^3J_{3A,4A}$ $^3J_{3A,4B}$ 6
H-3B	1.98	2.17	2.10	2.12	m *(cis)*	$^2J_{3B,3A}$ 13.0; $^3J_{3B,2}$ 3.0; $^3J_{3B,4A}$ $^3J_{3B,4B}$ 6
H-4A	2.76	2.50	2.95	3.01	overlapping	$^2J_{4A,4B}$ 18; $^3J_{4A,3B}$ 8; $J_{4A,3A}$ 6
H-4B	(overlap)	2.50	2.95	2.98	d/dd	$^2J_{4B,4A}$ 18; $^3J_{4B,3B}$ 6; $^3J_{4B,3A}$ 6
H-1′A	3.5	3.15	3.45	3.48	dd *(trans)*	$^2J_{1'A,1'B}$ 15.2; $^3J_{1'A,2'}$ 8.6
H-1′B	(overlap)	3.45	3.87	3.94	dd *(cis)*	$^2J_{1'B,1'A}$ 15.2; $^3J_{1'B,2'}$ 2.2
H-2′	3.72	4.03	3.99	4.05	td	$^3J_{2',1'A}$ 8.8; $^3J_{2',1'B}$ 2.2; $^3J_{2',3'}$ 5
H-3′		3.80	3.72	3.77	m	
H-4′			3.72			
H-5′	3.5	3.75	3.69	3.87	overlapping	
H-6′A	(overlap)		3.63		m	
H-6′B			3.82			
$CONH_2$	9.15					
	8.65					
>NH	6.09					
OH (2)	4.67					
OH (2)	4.50					

[a]Comparison of the ^{1}H-n.m.r. data for natural agropine reported by Coxon[5] at 350 MHz in D_2O with the 270-MHz data for synthetic agropine (6) and its acyclic precursor (5). Chemical-shift data in deuterated dimethyl sulphoxide for synthetic agropine (6) are also presented. Assignments and coupling constants were obtained by homonuclear decoupling experiments. The identity of the synthetic and natural agropine spectra at 270 MHz is shown in Fig. 4 and only the synthetic data are tabulated.

fact[3] that those strains of bacteria which induce the tumorous galls, can degrade the corresponding opines produced in the gall tissue. In this manner, it was possible to show[11] that the acyclic reductive conjugate (**4**) derived from D-mannose and L-glutamic acid was utilised by those strains that also utilise agropine as the sole carbon and nitrogen source, whereas the analogous products derived from D-glucose and D-galactose were not utilised. In addition it was found[11] that compound **4** was indistinguishable chromatographically and electrophoretically from a minor polyol-opine present in fresh extracts of gall tissue containing agropine.

Recognition of the presence of a second nitrogen atom in the structure of agropine prompted us to repeat the reductive condensation with D-mannose and L-glutamine to obtain the acyclic polyol condensation-product **5** in 21% yield. This acyclic product was also found to be indistinguishable electrophoretically from a second, minor, polyol opine present in fresh extracts of gall tissue containing agropine. Lactonisation of the acyclic polyol-opine **5** was achieved at room temperature with trifluoroacetic anhydride and the extraneous trifluoroacetyl groups were then removed by brief hydrolysis (0.5 h at 100°). The desired lactone **6** was isolated by cation-exchange and partition chromatography as a colourless, crystalline product in 19.7% yield. Assignment of the six-membered lactone structures **6** to the cyclisation product was inferred from the consumption of three mol of periodate and production of one mol of formaldehyde per mol of lactone oxidised. Once the final impurities present in natural agropine had been removed by the cellulose-column procedure, it ceased to be "intractable"[5] and crystallised. Crystalline natural agropine was found to be indistinguishable from the synthetic N^2-(1′-deoxy-D-mannitol-1′-yl)-L-glutamine 1,2′-lactone (**6**) in all properties examined, including m.p., mixed m.p., optical

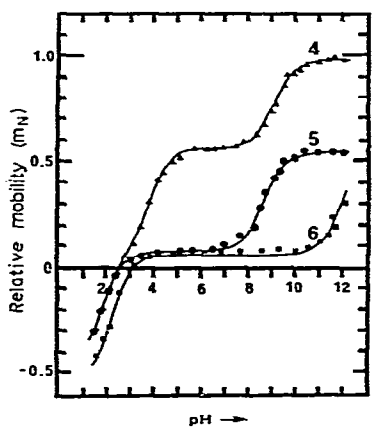


Fig. 5. pH–Relative mobility profiles for synthetic agropine (**6**) and its acyclic precursor (**5**) derived from D-mannose and glutamine. The related, acyclic opine (**4**) derived from D-mannose and glutamic acid (**4**) is also shown. Mobilities are expressed relative to *m*-nitrobenzenesulphonate (M_N 1.0) and glycerol (M_N 0.0) as arbitrary standards of unit and zero mobility, respectively. Solid lines represent recalculated[7] mobilities using derived pK_a values and single-species relative mobilities in Table I.

TABLE III

^{13}C-N.M.R. DATA[a]

Compound	*pD*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-1′*	*C-2′*	*C-3′*	*C-4′*	*C-5′*	*C-6′*
Natural agropine (Coxon[5])	acid	173.7[a]	70.9	26.7	32.6	176.4[a]	52.1	70.6[b]	73.4[b]	73.2[b]	71.4[b]	65.8
Natural agropine (this work)	6.2	178.02	68.78[a]	25.40	31.0	171.13	50.19	69.62[a]	71.49	71.49	71.37	64.01
Synthetic agropine (6)	6.2	178.05	68.74[a]	25.39	30.99	171.10	50.18	69.59[a]	71.46	71.46	71.35	64.01
Mannitol (Angyal and Le Fur[13])	neutral						64.6	72.2	70.7	70.7	72.2	64.6
L-Glutamine (Voelter[24])	acid	179.5	52.2	28.3	33.0	174.5						
5-Oxoproline (3) (Coxon[5])	acid	182.6	56.8	25.2	30.1	176.8						
Voelter[24]	6.3	180.2	58.35	25.35	29.65	181.2						
Agropinic acid (2) (Coxon[5])	acid	182.0	64.9	25.4	31.9	178.4	48.6	72.1	73.4	73.2	71.5	65.7

[a]Comparative ^{13}C-n.m.r. data in D_2O for natural and synthetic agropine and related compounds. Signals having the same alphabetic superscript may be transposed within a row. All carbon atoms are numbered as if they were moieties derived from glutamine or mannose, and carbon atom numbers for mannose are primed, as in the agropine formula in Fig. 4.

rotations from 589–365 nm, i.r. spectra (Fig. 3), 270-MHz ^{1}H n.m.r. (Fig. 4), 67.9-MHz ^{13}C n.m.r. (Table II), f.d. and e.i.m.s., electrophoretic mobilities from pH 1.5–12.0 (Figs. 1 and 5), paper chromatographic R_F values, and biological utilisation experiments.

Melting points and optical rotations measured on analytically pure synthetic agropine (m.p. 177–178°, $[\alpha]_D^{25}$ +44.5°) and natural agropine (m.p. 176–177°, $[\alpha]_D^{25}$ +44.2°) are substantially higher than the data (m.p. 129°, $[\alpha]_D^{25}$ +19.5°) reported by Firmin and Fenwick[4] for natural agropine. However, until crystallization of agropine had been achieved in the present study, broad and low melting points and low optical rotations were also noted.

The pH–mobility profiles shown in Figs. 1 and 5, or the derived pK_a values (Table I), provide useful data for the choice of appropriate pH (or pD) conditions for physical measurements. For this reason, optical rotations and n.m.r. measurements were made on the uncharged agropine molecule at either pH 5.6 in water or[12] pD (=pH meter reading + 0.4) = 6.2 in D_2O. After allowing for differing assignments, there is reasonable agreement (Table II) for the ^{1}H-n.m.r. data obtained at 270 MHz in the current work and the 350-MHz data of Coxon *et al.*[5]. The ^{13}C-n.m.r. data of Coxon *et al.*[5] were obtained in acidified D_2O of unspecified pD and are therefore not directly comparable with the data for uncharged natural and synthetic agropines measured at pD 6.2 (Table III).

Angyal and LeFur[13] have shown that, apart from enantiomorphs, in D_2O each and every alditol from ethylene glycol to the hexitols produces a unique ^{13}C-n.m.r. spectrum, which therefore provides a valuable criterion for identity (Table III), as do the ^{1}H-n.m.r. data (Fig. 4). Comparison (Table II) of the ^{1}H-n.m.r. data for the acyclic agropine precursor **5** and synthetic agropine **6** shows that lactonisation deshields the following protons: H-2 (0.72 p.p.m.), H-3A (0.35 p.p.m.), H-4A, H-4B (0.45 p.p.m.), H-1'A (0.32 p.p.m.) and H-1'B (0.43 p.p.m.); the chemical shifts for the remaining protons are not significantly affected. A possible explanation may be derived from inspection of a model for agropine, which shows an intramolecular hydrogen-bond from an equatorial imino hydrogen atom to the carbonyl oxygen atom of the amide group. Anisotropy of the amide carbonyl group in the flexible, seven-membered ring thus formed would deshield all protons brought into the appropriate proximity to it, by flexions of the seven-membered ring, except for proton H-3B, which remains remote from the amide carbonyl group in all conformations of the seven-membered ring.

An important consequence of the hydrogen bond to the amide carbonyl group is that there would be increased acidity for the first proton dissociation of the amide and would thus afford a rational explanation for the weak acidity (pK_a 12.04) of agropine. The extraordinarily weak basicity (pK_a 2.17) of agropine **6** compared with its acyclic precursor **5** (pK_a 8.64) also requires comment. It is known that morpholine (pK_a 8.3)[14] is significantly lower in basicity than its closest mono-oxygenated acyclic analogue *N*-(hydroxyethyl)ethylamine (pK_a 9.9)[15]. Furthermore, esterification of glutamic acid (pK_a 10.0)[14] to the diethyl ester (pK_a 7.0)[14], exhibits a decrease of

three pK units. If we assume that both of these effects are operative and additive then, in the case of the heterocyclic-lactonisation of 5, these two effects would account for much (4.6 pK_a units) of the observed decrease in basicity (6.4 pK_a units). Nevertheless, a closer study of all the factors involved is warranted.

Biosynthesis of opines via the reductive conjugation of an L-amino acid and a carbonyl moiety has been reported[16], and it seems reasonable to expect that the acyclic agropine precursor 5 arises similarly from L-glutamine and D-mannose. In addition, existence of the lactone ring in agropine suggests there is also an enzymic cyclisation step; whether or not these plant enzymes are encoded by the bacterial T-DNA or are merely "turned on" remains to be established.

Finally, structure elucidation of agropine has demonstrated (*a*) the utility of an extremely simple technique (pH–electrophoretic mobility profile) to reveal and quantify ionization phenomena, (*b*) the virtues of f.d.m.s. for obtaining protonated molecular species from labile natural products, and (*c*) the economy of effort achieved by biosynthetic reasoning and biological utilisation studies to select the correct structure (6) for synthesis from among its 32 possible stereoisomers.

EXPERIMENTAL

Instrumental procedures. — Mass spectrometry was conducted with a Varian MAT 311A instrument equipped with a combined e.i.–f.d. source and the data were processed with a Varian MAT Spectrosystem 100. Field emitters were made from 10-μm tungsten wire and were of the high-temperature type[17,18]. Low-resolution f.d. spectra were taken by repetitive scanning over the appropriate mass range, while the emitter heating-current was raised in a linear manner. Optimal results were obtained with an initial emitter-current of 15 mA and an increment rate of 0.21 $mA.sec^{-1}$. The source temperature was 90°, cathode voltage 5–6 kV, and accelerating voltage 3 kV. High-resolution mass measurement under f.d. conditions was by a modification of the Varian MAT technique[19]. Low resolution e.i. spectra were obtained by retracting the sample on the emitter from the electron beam. Mass-spectral comparisons were made with a Hewlett–Packard library-search program on a 9825A calculator. The library-search program computes and ranks the significance (significance = mass × abundance) for each mass in a spectrum, and the ten most significant masses are then compared *via* a "similarity index" with the ten most significant masses in another reference spectrum. The similarity index is an algorithm: $= (\Sigma_m A.a.)/(\Sigma_m A^2 \cdot \Sigma_m a^2)^{\frac{1}{2}}$, where m is a mass in either the unknown or the reference sets of the most significant masses, and A and a are the corresponding abundance values of the mass m in the unknown and reference set, respectively. The values of the similarity index range from 0.0 for a perfect mismatch to 1.0 for a perfect match.

Optical rotations were measured in water with a 1-dm cell and a Perkin–Elmer Model 141 polarimeter. I.r. spectra were obtained as 0.5% w/w sample concentrations in potassium chloride discs with a Perkin–Elmer model 237 grating spectrophotometer. N.m.r. data were obtained with 25-mg samples in D_2O or Me_2SO-d_6 (0.5 mL)

by Dr. A. Jones of the Australian National N.M.R. Centre, A.N.U., Canberra, with a Bruker HX270 instrument at 25°, measuring proton nuclei at 270 MHz and carbon-13 nuclei at 67.89 MHz in the pulse (Fourier) mode, using 5–15-mm sample tubes. Chemical shifts are referenced to tetramethylsilane by using either sodium 4,4-dimethyl-4-silapentanoate-d_4 (^{1}H data), 0.0 p.p.m., or 1,4-dioxane (^{13}C data), 67.40 p.p.m., as internal standards for primary measurements. Melting points were obtained with a Kofler hot-stage microscope. Elemental analyses were performed by the Australian Microanalytical Service, Port Melbourne, Victoria.

Electrophoretic and chromatographic procedures. — pH–Relative electrophoretic mobility profiles were obtained with glycerol and *m*-nitrobenzenesulphonate as standards of zero and unit mobility in buffers containing 0.05M oxalate ions on Whatman 3 MM paper as described previously[7], with the exception that *p*-hydroxybenzoate buffers were substituted for pyrophosphate buffers in the range pH 8.3–9.5 to minimise discontinuities due to ionic-strength changes in the range pH 8–10. The u.v. absorption due to the hydroxybenzoate buffer was removed after drying by repeated (3×) extraction with fresh solutions of 9:1 (v/v) acetone–M formic acid followed by oven drying at 110°.

Preparative electrophoresis (1 h at 2500 V) was performed with a volatile buffer (pH 1.7) that was M with respect to acetic acid and 0.75M with respect to formic acid; samples were applied as a band (0.3 mg/cm) to Whatman 3 MM paper (15 × 5.7 cm) with reference standards at the edges. For identity purposes, a complexing buffer of 0.05M sodium tetraborate pH 9.2 was also used; in this case 2′-deoxyadenosine was used as a zero-mobility marker in place of glycerol. Orange G (O.G.) was employed as a convenient standard for relative mobility measurements, but it is not suitable[7] for pH–mobility profiles.

Paper chromatography was performed with Whatman No. 1 filter paper by the descending technique using the following solvent-systems: *A*, 10:4:3 (v/v/v) ethyl acetate–pyridine–water; *B*, 4:1 (v/v) acetone–water; and *C*, 4:1 (w/w) phenol–water. Polyols were detected with the silver nitrate–ethanolic sodium hydroxide sequence-dip[20]; 5-oxoproline was also detectable as a white spot with the latter reagent. Both amino and imino compounds were detected with a dip of 0.25% (w/v) ninhydrin in acetone when papers were heated for 15 min at 110°; contrary to an earlier report[5], agropine and also the acyclic polyol-imino acids **4** and **5** appear as weak blue spots.

Periodate oxidation. — Polyol oxidations[21] were carried out at 25° in the presence of an excess of 85mM sodium metaperiodate prepared in 0.1M sodium acetate buffer (pH 4.7). Periodate consumption was monitored by the decrease in absorption at 300 nm in a 1-cm cuvette. Under these conditions, a control reaction containing 17mM erythritol was completely oxidised in 5 min with a ratio[21] of 3.01 mol of sodium metaperiodate consumed per mol of erythritol. Crystalline samples of synthetic and natural agropine at 19mM concentrations were completely oxidised at indistinguishable rates within 10 min and respectively consumed 2.97 and 2.93 mol periodate per mol of agropine; 0.99 and 1.01 mol of formaldehyde[22] were also produced. Agropinic acid derived independently from the ammonolysis of natural agropine and the acyclic

synthetic agropine precursor (**5**) respectively consumed 3.82 and 3.81 mol of periodate per mol of agropinic acid and also produced 0.98 and 0.97 mol of formaldehyde within 10 min. In addition, 5-oxoproline was detectable by electrophoresis as one of the reaction products, as reported by Coxon *et al.*[5].

Periodic oxidation of the acyclic agropine precursor **5** was not straightforward and was marked by a rapid rise (<1 min) in absorption at 300 nm to an $A_{300} = 1.45$ which slowly (6 h) decayed to $A_{300} = 0.17$, corresponding to a consumption of 4.3 mol of periodate per mol of **5** and at 30 min only 0.75 mol of formaldehyde[22] was produced. Apparently, the zwitterion of **5** forms a complex with periodate at pH 4.7, which is only slowly oxidised, by comparison with agropine, which is uncharged (Fig. 1) at pH 4.7.

Isolation of agropine. — Natural agropine was isolated from stem tumours (6–10 weeks) induced by inoculation of *Briophyllum daigremontianum* × *tubiflora* (formerly *Kalanchoe*) with *A. tumefaciens* strain A281[3,23] which harbours the pTi 542 plasmid. Stem tumours (530 g) were macerated in 7:3 (v/v) ethanol–water (5 mL per g), with a high-speed homogeniser and then filtered. The filtrate was concentrated *in vacuo* to 700 mL and particulate material was removed by centrifugation. The supernatant solution was applied to a column (50 × 3.5 cm) of 100–200 mesh Dowex 50W (× 2), H^+ form, and eluted with water (2.5 L). Stepwise elution with 0.25M pyridine–M acetic acid buffer (1 L), followed by 0.5M pyridine–M acetic acid buffer eluted the agropine in the second stage. Fractions (100 mL) were individually examined by electrophoresis with the pH 1.7 formic–acetic acid system and fractions 17 and 18, containing cationic ($M_{O.G.}$ −0.46) material, which yielded a strong positive polyol reaction with the silver nitrate–ethanolic sodium hydroxide sequence, were combined. After evaporation at 37°, the residual buffer components were removed by distillation of water from the residue until constant weight (1.35 g) was achieved. This yellow oil represented 0.25% on a fresh-weight basis. Electrophoresis at pH 1.7 indicated the presence of other ninhydrin-positive, polyol-negative components in the crude agropine fractions as well as in the authentic[3,4] reference samples. At this point, small samples (4 mg) were purified to electrophoretic homogeneity by preparative electrophoresis and used for f.d.m.s. (Fig. 2) and pH–mobility profile studies (Fig. 1). The bulk of the crude agropine (1.26 g) was extracted with 4:1 acetone–water (100 mL) and applied to a column (46 × 1.8 cm) of powdered cellulose (80 g) packed in 4:1 acetone–water. The column was eluted (5 mL/min) with the same solvent and fractions (200 mL) were collected; aliquots from each fraction were checked for the presence of agropine by electrophoresis at pH 1.7. Electrophoretically homogeneous agropine was eluted in fractions 8 and 9 (total weight 201 mg); fraction 7 (47.5 mg) also contained agropine but was heterogeneous. Homogeneous agropine (200 mg) was dissolved in methanol (15 mL) and centrifuged; the supernatant solution was evaporated to a glass in a stream of nitrogen and trituration with a glass rod and 1-propan-ol (1.5 mL) induced crystallization. The excess of solvent was removed by filtration and the colourless, crystalline residue was dried to constant weight (164 mg) m.p. 175–176° (dec.), $[\alpha]_{589}^{25}$ +44.2, $[\alpha]_{546}^{25}$ +55.5, $[\alpha]_{436}^{25}$ +119.0, and $[\alpha]_{365}^{25}$

+246.2° (c 0.92, pH 5.6); i.r. Fig. 3; ^{1}H-n.m.r. Fig. 4; ^{13}C-n.m.r. Table III; f.d. data Fig. 2; e.i. data *m/z* 275 (11) $(M - 17)^+$, ten most significant peaks and their abundances 257 (91), 245 (52), 230 (82), 202 (95), 172 (76), 143 (66), 142 (82), 130 (94), 98 (87), and 84 (100).

Anal. Calc. for $C_{11}H_{20}N_2O_7$: C, 45.20; H, 6.90; N, 9.58. Found: C, 45.13; H, 6.81; N, 9.14.

Agropinic acid (**2**). — Agropine (22.6 mg) in 3M ammonium hydroxide (5 mL) was heated for 1.5 h on a steam bath under reflux. Electrophoresis showed that the bulk of the material had the electrophoretic properties (Fig. 1) of agropinic acid and a small amount of a product having electrophoretic properties (Fig. 5) of the acyclic agropine precursor **5**. The excess of ammonia was removed by evaporation and the oily residue was dissolved in water (2 mL) and passed through a column (6.5 × 1.2 cm) of Dowex 50 (× 2), H^+ form. The eluate was evaporated and the residue crystallised as rods from methanol (0.3 mL); weight 15.2 mg (67%) m.p. 165–166, $[\alpha]_{589}^{25}$ +17.9, $[\alpha]_{546}^{25}$ +22.8, $[\alpha]_{436}^{25}$ +52.6, and $[\alpha]_{365}^{25}$ 114.6° (*c* 0.4, M hydrochloric acid). The i.r. spectrum showed a strong *N,N*-disubstituted amide carbonyl band at 1658 cm^{-1} with shoulders at 1690 cm^{-1} (CO_2H) and 1600 cm^{-1} (CO_2^-); single-bond (C-O) stretching frequencies at 1230, 1062, 1057, 1037 and 1028 cm^{-1} are also in accord with the structure proposed by Coxon *et al.*[5]. The pH–mobility profile (Fig. 1) is also consistent with the N^2-substituted-5-oxoproline (**2**).

Anal. Calc. for $C_{11}H_{19}NO_8$: C, 45.05; H, 6.53; N, 4.78. Found: C, 44.99; H, 6.51; N, 4.70.

Ammonolysis (3M ammonium hydroxide for 8 h at 95°) of the synthetic acyclic precursor (**5**) of agropine yielded a product having m.p. 166–167°, indistinguishable from agropinic acid.

N^2-(*1'-Deoxy*-D-*mannitol-1'-yl*)-L-*glutamine* (**5**). — L-Glutamine (14.6 g, 100 mmol), sodium hydrogencarbonate (8.4 g, 100 mmol) and D-mannose (27.0 g, 150 mmol) were dissolved in water (350 mL) and to the stirred solution, sodium cyanoborohydride (7.88 g, 125 mmol) was added and stirring was continued for 17 h at 30° in a fume hood. The mixture was decomposed (HCN!) by slow addition of Dowex 50 (× 2) (H^+) resin until the supernatant solution gave a negative ninhydrin test. When the bulk of the gaseous products (HCN, CO_2) had ceased being evolved, the resin was packed into a column and washed with water (5 bed volumes) and finally eluted in fractions (100 mL) with cold (5°) 0.5M ammonium hydroxide (2.5 L), at which stage the eluate was pH 11. Aliquots of each fraction were examined by electrophoresis at pH 1.7 and fractions containing the cationic ninhydrin positive polyol having a relative mobility $M_{O.G.} = -0.26$ were combined and concentrated under diminished pressure at 37° to yield an oil (15.6 g, 50.3%); trituration with a glass rod in the presence of 4:1 (v/v), acetone–water (300 mL) induced crystallisation. Two recrystallisations from water (200 mL) plus acetone (800 mL) gave needles (6.5 g, 20.8%), m.p. 173–175°. One more crystallisation gave an analytical sample (**5**), m.p. 185–186°, and further recrystallisation did not raise the m.p.; $[\alpha]_{589}^{25}$ +9.0, $[\alpha]_{546}^{25}$ +10.7, $[\alpha]_{436}^{25}$ +18.4, and $[\alpha]_{365}^{25}$ +29.6° (*c* 1.02, pH 5.6); the i.r. spectrum

showed strong amide carbonyl absorbances at 1655 and 1565 as well as carboxylate absorption at 1602 cm^{-1}. Single-bond (C-O) absorptions were observed at 1318, 1102, 1075, 1060, 1028, and 1008 cm^{-1}; ^{1}H-n.m.r. compare Table II, m.s. (Field desorption): 311 $(M_1 + H)^+ \xrightarrow{-17}$ 294 $(M_2 + H)^+ \xrightarrow{-18}$ 276 $(M_3 + H)$, also ions at 333 $(M_1 + Na)^+$ and 316 $(M_2 + Na)^+$; no $(M_3 + Na)^+$.

Anal. Calc. for $C_{11}H_{22}N_2O_8$: C, 42.58; H, 7.15; N, 9.03. Found: C, 42.86; H, 7.03; N, 8.75.

The product was electrophoretically indistinguishable from a minor component of the gall-tissue extract used for the isolation of agropine, but no further identity criteria have been established.

N^2-(*1'-Deoxy*-D-*mannitol-1'-yl*)-L-*glutamine*, *1-2'-lactone* (**6**). — The acyclic N^2-polyol substituted glutamine (**5**, 620 mg, 2 mmol) was suspended in cold (0°) 1,4-dioxane (10 mL) and stirred magnetically during the dropwise addition of trifluoroacetic anhydride (3.5 mL, 11.1 mmol) during 15 min. Starting material dissolved completely after stirring for 2 h at 30°. The mixture was cooled (0°) and distilled water (4 mL) was slowly added to maintain the exothermic hydrolysis below 15°. Finally, the protective trifluoroacetyl groups were removed by heating the mixture for 30 min on a steam bath; progress of the hydrolysis was monitored by electrophoresis at pH 1.7, which showed hydrolysis of the starting material ($M_{O.G.}$ −0.46) and a second unidentified component ($M_{O.G.}$ −0.88). The mixture was applied to a column (40 × 3.9 cm) of 50–100 mesh Dowex 50 (×2) (H^+) resin at 5° and the neutral and acidic species eluted with water (400 mL) were discarded. The column was then eluted at 1 mL/min with 0.5M ammonium hydroxide and fractions (15 mL) were collected. Electrophoresis of aliquots from individual fractions showed material corresponding to agropine in fractions 125–132 and the second component ($M_{O.G.}$ −0.88) in fractions 126–140. Fractions (125–132) corresponding to agropine were combined and evaporated to dryness at 37° (0.39 g). The mixture was separated by a column (40 × 3.9 cm) of Dowex 50 (×2) (NH_4^+), which was packed in and eluted with a 0.2M ammonium formate buffer, pH 2.5. After 155 fractions (15 mL), the pH of the eluant was raised to 3.4 with ammonia, whereupon material corresponding to agropine was eluted in fractions 206–219. Formate buffer salts were removed by adsorption of the cations onto a column (23 × 1.8 cm) of Dowex 50 (×2) (H^+) resin and elution with water (130 mL) followed by 0.5M ammonium hydroxide, whereupon material corresponding to agropine was eluted with the pH front. The appropriate fractions were combined and evaporated to a syrup at 30°, which solidified to an almost white, amorphous solid (0.25 g, 42.8%) whereupon the residual moisture was azeotropically removed by evaporation with 1-propanol (15 mL). Attempts to crystallise the product were unsuccessful, and the crude material was dissolved in 4:1, acetone–water (30 mL) and applied to a column (31.5 × 3.8 cm) of powdered cellulose (90 g) packed and eluted with 4:1 acetone–water; fractions (75 mL) were collected and aliquots were examined by electrophoresis at pH 1.7. Electrophoretically homogeneous material corresponding to agropine ($M_{O.G.}$ −0.46) in fractions 24–35 were concentrated at 35° and azeotropically dried by evaporation

with 1-propanol (2 × 10 mL) to yield an almost colourless solid (0.21 g, 36.1%); m.p. softened at 97–110°, m.p. 135–170°. Crystallisation was achieved by dissolving the product in hot methanol (70 mL), filtering, and concentrating by evaporation to ~3 mL; rosettes of needles were induced by scratching the vessel with a glass rod and keeping the solution for 24 h at room temperature. The crystals were filtered off, washed with methanol (2 × 0.1 mL), 1:1 1-propanol-methanol (3 × 0.1 mL) and dried (0.115 g, 19.7%), m.p. 177–178°; the mixed m.p. (176–178°) with crystalline, natural agropine (m.p. 175–176°) was not depressed. Optical rotations were $[\alpha]_{589}^{25}$ +44.5, $[\alpha]_{546}^{25}$ +55.6, $[\alpha]_{436}^{25}$ +119.0, and $[\alpha]_{365}^{25}$ +246.2° (*c* 1.1, pH 5.6); i.r. (see Fig. 3); ^{1}H-n.m.r. data (see Fig. 4 and Table II); ^{13}C-n.m.r. data (see Table III); e.i.m.s. data: *m/z* 275 (10, M − 17), ten most significant peaks and their abundances: 257 (62), 245 (43), 230 (81), 202 (87), 172 (84), 143 (82), 142 (88), 130 (94), 98 (80), and 84 (100); high-resolution f.d. data $C_{11}H_{21}N_2O_7$, $(M + H)^+$ requires 293.1349, found: 293.1353 ± 0.006. The low-resolution f.d. behaviour was indistinguishable from that of crystalline, natural agropine. Other identity criteria respectively for natural and synthetic agropine include chromatographic R_F values: solvent *A* 0.09, 0.1; *B* 0.09, 0.09; and *C* 0.76, 0.75; electrophoretic mobilities in 0.05M sodium tetraborate ($M_{O.G.}$ 0.61, 0.61); and a similarity index of 0.99 for the ten most significant peaks in the e.i.m.s. Under conditions described previously[11], the natural and synthetic agropine **6**, as well as the acyclic precursor(s) were fully utilised by the pathogenic *A. radiobacter* transconjugant strain TC3, which harbours[23] the Ti plasmid pTi 542, whereas none of the samples were utilised by the nonpathogenic *A. radiobacter* strain C58C1.

ACKNOWLEDGMENTS

The financial support of the Australian Research Grants Committee is gratefully acknowledged. This work was supported by the French C.N.R.S. contracts ATP3363 and AIO37250.

REFERENCES

1 M. Drummond, *Nature (London)*, 281 (1979) 343–347.
2 A. Petit, Y. Dessaux, and J. Tempé, *Proc. Int. Conf. Plant Path. Bact., 4th*, Angers (1978) INRA edn., (1978) pp. 143–152.
3 P. Guyon, M. D. Chilton, A. Petit, and J. Tempé, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 2693–2697.
4 J. L. Firmin and G. R. Fenwick, *Nature (London)*, 276 (1978) 842–844.
5 D. T. Coxon, A. M. C. Davies, G. R. Fenwick, R. Self, J. L. Firmin, D. Lipkin, and N. F. James, *Tetrahedron Lett.*, (1980) 495–498.
6 D. A. Tepfer and J. Tempé, *C. R. Acad. Sci., Ser. D*, 292 (1981) 153–156.
7 M. E. Tate, *Biochem. J.*, 195 (1981) 419–426.
8 A. C. Chibnall and R. G. Westall, *Biochem. J.*, 26 (1932) 122–132.
9 H. B. Vickery, G. W. Pucher, H. E. Clark, A. C. Chibnall, and R. G. Westall, *Biochem. J.*, 29 (1935) 2710–2720.
10 J. B. Gilbert, V. E. Price, and J. P. Greenstein, *J. Biol. Chem.*, 180 (1949) 209–218.

11 J. TEMPÉ, P. GUYON, A. PETIT, J. G. ELLIS, M. E. TATE, AND A. KERR, *C. R. Acad. Sci., Ser. D*, 290 (1980) 1173–1176.
12 P. K. GLASOE AND F. A. LONG, *J. Phys. Chem.*, 64 (1960) 188–190.
13 S. J. ANGYAL AND R. LEFUR, *Carbohydr. Res.*, 84 (1980) 201–269.
14 D. D. PERRIN, *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London, 1965, pp. 196, 374, 375.
15 G. DOUHÉRET AND J. C. PARIAUD, *J. Chim. Phys.*, 59 (1962) 1013–1020.
16 L. A. B. M. OTTEN AND R. A. SCHILPEROORT, *Biochim. Biophys. Acta*, 527 (1978) 497–500.
17 H. D. BECKEY, E. HILT, AND H. R. SCHULTEN, *J. Phys. E*, 6 (1973) 1043–1044.
18 H. KAUFFMANN, U. RAPP, AND K. H. MAURER, *Mass Spectrom. Conf., 7th*, Florence, Aug. 30–Sept. 3, 1976, poster presentation.
19 B. SOLTMANN, C. C. SWEELEY, AND J. P. HOLLAND, *Anal. Chem.*, 49 (1977) 1164–1166.
20 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
21 J. S. DIXON AND D. LIPKIN, *Anal. Chem.*, 26 (1954) 1092–1093.
22 D. J. HANAHAN AND J. N. OLLEY, *J. Biol. Chem.*, 231 (1958) 813–828.
23 D. SCIAKY, A. MONTOYA, AND M. D. CHILTON, *Plasmid*, 1 (1978) 238–253.
24 W. VOELTER, S. FUCHS, R. H. SEUFFERT, AND K. ZECH, *Monatsh. Chem.*, 105 (1974) 1110–1135.