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L,L-SUCCINAMOPINE: AN EPIMERIC CROWN GALL OPINE*

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Key Word Index—*Agrobacterium tumefaciens*; crown gall; opine; succinamopine; leucinopine.

Abstract—Alfalfa tumour incited by *Agrobacterium tumefaciens* strain A281, carrying the tumour inducing plasmid pTi Bo542, synthesizes agropine and related mannityl opines. In addition it contains a small amount of leucinopine and large quantities of a new opine here identified as N-[(1*S*)-1-carboxy 2-carbamoylethyl]-(*S*)-glutamic acid. This new opine, L,L-succinamopine, is the L^{glu} epimer of the succinamopine previously isolated from tumours incited by pTi AT181 and related strains. The latter opine should now be designated D,L-succinamopine. This is the first example of the natural occurrence of epimeric opine structures.

INTRODUCTION

Crown gall tumours are incited by virulent *Agrobacterium tumefaciens* strains on susceptible dicot host plants. Tumour tissue synthesizes novel small M_r metabolites called 'opines' whose structures are specifically determined by the inciting strain [1]. Large plasmids called Ti (tumour-inducing) plasmids carry the virulence trait [2–4] and confer opine synthetic specificity [5, 6]. The Ti plasmids also confer on the pathogen the ability to catabolize the same opines synthesized in the corresponding tumour [5, 6]. A DNA transfer mechanism is responsible for tumour induction and opine synthesis in tumour cells: a small part of the virulence plasmid, called T-DNA (transferred DNA) is incorporated into tumour cell chromosomal DNA [7–11].

Three major structural families of opines have been identified: phosphorylated sugars [12], mannityl opines [13–16] and imino diacids [17–28]. The known imino diacid opines appear to derive from L-amino acids reductively conjugated to pyruvic acid or 2-ketoglutaric acid. Pyruvic acid derived opines are conjugates of the basic L-amino acids: lysopine from lysine [17, 18], octopine from arginine [19, 20], histopine from histidine [21] and octopinic acid from ornithine [20, 22]. Ketoglutaric acid derived glutamyl opines are conjugates of the L-amino acids: nopaline from arginine [23], ornaline from ornithine [24], succinamopine (**1**) from asparagine [25, 26] and leucinopine from leucine [27, 28]. Stereochemistry of all pyruvic derived opines is D^{ala}, $L^{amino\ acid}$. While most ketoglutaric derived glutamyl opines are similarly of D^{glu}, $L^{amino\ acid}$ stereochemistry, leucinopine has been proven to have L^{glu}, L^{leu} stereochemistry [28].

Leucinopine is a poor nutritional substrate for the bacterial strains eliciting its synthesis in tumours [28], and it is not the major iminodiacid opine in all such tumours. We here report that a new major opine in tumours synthesizing leucinopine shares its unusual L^{glu} configuration and is an excellent carbon source for the inciting *Agrobacterium* strain. This new opine is the L^{glu} epimer of the known opine succinamopine. In this and previous publications on glutamyl opines possessing two centres of asymmetry about an iminodiacid group [26, 28] we refer to the chirality of the glutamyl centre first and then to the chirality of the other amino acid centre.

The iminodiacid fraction of A281 alfalfa tumour extract contains two acidic substances detectable by chelation of silver nitrate. These substances exhibit the diagnostic characteristics of opines: they are absent from normal alfalfa tissue and they are catabolized by the pathogenic *A. tumefaciens* strain A281 but not by the corresponding plasmidless strain A136 [28].

Electrophoretic mobility of the major iminodiacid opine

The major iminodiacid opine of A281 alfalfa tumour has electrophoretic mobility (u_{pic}) 1.82 at pH 8.3 and 2.10 at pH 10.8 and comigrates with succinamopine and its diastereoisomer in these buffers. At pH 2.8 the succinamopine diastereoisomers are resolved. The absolute configuration of these diastereoisomers has been elucidated previously by circular dichroism and by selective fermentation [26]. Synthetic D^{glu}, L^{asn}-succinamopine (**1**) and succinamopine isolated from A519 tobacco tumour have u_{pic} 0.43, while synthetic L^{glu}, L^{asn}-succinamopine (**2**) and the major opine isolated from A281 alfalfa tumour have u_{pic} 0.54. Mixtures of A281 tumour opine and synthetic L,L-succinamopine were unresolvable on long electrophoretic runs at pH 2.8. Thus the major opine in A281 alfalfa tumour has electrophoretic mobility indistinguishable from L,L-succinamopine in three different buffers, but clearly distinguishable from D,L-succinamopine at pH 2.8.

The lactam derived from the major A281 tumour opine has the mobility at pH 2.8 of synthetic L,L-succinopine lactam (**4**) (u_{pic} 0.60) and different from the mobility of D,L-succinopine lactam (u_{pic} 0.98).

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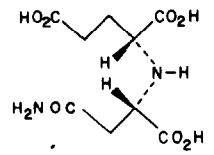
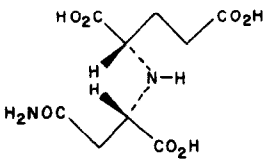


1. D,L-SUCCINAMOPINE



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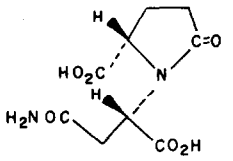
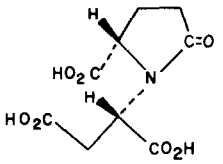


3. L,L-SUCCINAMOPINE LACTAM



4. L,L-SUCCINOPINE LACTAM

The minor opine from A281 alfalfa tumour has u_{pic} 0.58 at pH 2.8 and comigrates with leucinopine. The minor opine is catabolized by the same strains that catabolize leucinopine and not catabolized by strains that do not utilize leucinopine.

Spectroscopic characterization of the major iminodiacid opine

Fast atom bombardment (FAB) mass spectroscopic analysis of the iminodiacid fraction from A281 alfalfa tumour gave a protonated molecular ion at *m/z* 263.0876 (±0.3 mmu), corresponding to the molecular formula $C_9H_{14}N_2O_7$, an isomer of succinamopine. The proton NMR spectrum of the major iminodiacid opine in D_2O at pD 2 shows strongly coupled glutamyl beta and gamma methylene multiplets (δ2.27 and 2.63, 4H), an aspartyl beta methylene four-line ABX pattern (δ3.13, 2H) and two overlapping alpha proton multiplets (δ3.7–4.1, 2H).

Acid catalysed lactamization of the new opine from A281 tumour produced a mixture containing an intermediate detectable by electrophoresis at pH 2.8 as a silver nitrate chelating substance between the electrophoretic locations of L,L-succinamopine and L,L-succinopine lactam. The intermediate is presumably the lactam amide, L,L-succinamopine lactam (3). The FAB mass spectrum of samples withdrawn from the acidic solution over a period of several hr showed the sequential loss of the L,L-succinamopine quasi-molecular ion (M + H, *m/z* 263), appearance of the intermediate L,L-succinamopine lactam (*m/z* 245), and final appearance of L,L-succinopine lactam (*m/z* 246).

The circular dichroism (CD) of the new opine lactam at pH 1 had a positive extremum at 213 nm and exhibited a large positive shift on changing the pH to 5, with no further shift at pH 10. This CD behaviour is identical to that of synthetic L^{glu}, L^{asp}-succinopine lactam and the mirror image of the CD spectrum of D^{glu}, L^{asp}-succinopine lactam isolated from A519 tobacco tumour [26].

Fermentative characterization of the major iminodiacid opine

In liquid culture *A. tumefaciens* strain A281 completely catabolized the new iminodiacid opine and leucinopine within 72 hr. Strain A281 also rapidly catabolized synthetic L,L-succinamopine prepared by reduction of a mixture of L-asparagine and 2-ketoglutaric acid, but did not diminish the epimeric synthetic D,L-succinamopine in the same liquid medium within ten days (Table 1). A mixture of the L,L- and D,L-epimers was also able to serve as sole carbon source for normal growth of strain A281 on agar plates. In liquid culture strain A281 was unable to catabolize either the D^{glu}, D^{asn}- or the L^{glu}, D^{asn}-succinamopine diastereomers prepared from D-asparagine and 2-ketoglutaric acid. Therefore strain A281 is capable of catabolizing only one of the four diastereoisomers of succinamopine, namely the same diastereoisomer produced in tumours incited by this strain. Control strains A136 and A114 with no Ti plasmid and prototype octopine (A277) and nopaline (A208) strains catabolized none of the succinamopine diastereomers.

Surprisingly, succinamopine-type strains A518, A519 and A532 catabolized both D^{glu}, L^{asn}-succinamopine and L^{glu}, L^{asn}-succinamopine. It has been previously noted that these strains use the unnatural D^{glu}, L^{leu}-leucinopine as well as L,L-leucinopine [28], an opine not found in tumours incited by these strains. An unknown opine X has been noted in tobacco tumour incited by A519 [25]. Opine X is neither L,L-leucinopine nor L,L-succinamopine, for it is not catabolized by strains A281, A543 and AT1TF. It may be an opine of L,L configuration whose catabolase is responsible for the unexpected ability of strains A518, A519 and A532 to catabolize L,L diastereoisomers of leucinopine and succinamopine.

The lactam of L,L-succinamopine is not catabolized by any of the strains tested, including those that utilize L,L-succinamopine itself. In contrast, strains using D,L-succinamopine can catabolize the corresponding lactam.

DISCUSSION

All agropine/leucinopine type strains tested (A281, A543, AT1TF) were able to catabolize only the isomers of leucinopine and succinamopine possessing the L^{glu}, $L^{amino\ acid}$ configuration. It is possible that a single L,L-dehydrogenase of low side chain specificity is responsible for cleavage of both of these opines. Mannityl opine degrading enzymes have been shown to be similarly promiscuous in accepting analog substrates as long as the chirality around the imino nitrogen is preserved [29].

A substantial family of glutamyl-type opines is now

Table 1. Catabolism of diastereomeric succinamopines and lactams*

Bacterial strain	Plasmid† type	Succinamopine diastereomers‡				Succinopine lactams‡			
		D,L§	L,L‖	L,D	D,D	D,L	L,L	L,D	D,D
A114	none	−	−	−	−	−	−	−	−
A136	none	−	−	−	−	−	−	−	−
A208	NOP	−	−	−	−	−	−	−	−
A277	OCT	−	−	−	−	−	−		
A281	AGR/LOP	−	+	−	−	−	−		
AT1TF	AGR/LOP	−	+	−	−	−	−		
A543	AGR/LOP	−	+	−	−	−	−		
A518	SAP	+	+	−	−	+	−		
A519	SAP	+	+	−	−	+	−	−	−
A532	SAP	+	+	−	−	+	−		

*Compound catabolized completely within 72 hr, +; not catabolized within one week, −.
†NOP, Nopaline; OCT, octopine; AGR, agropine; LOP, leucinopine; SAP, succinamopine.
‡Configuration of glutamyl centre specified first, followed by aspartyl centre.
§Identical catabolism obtained with opine isolated from A519 tobacco tumour.
‖Identical catabolism obtained with opine isolated from A281 alfalfa tumour.

recognized: nopaline, ornaline, D,L-succinamopine, leucinopine, agropine, mannopine and mannopinic acid in addition to L,L-succinamopine described here. All glutamyl opines are known to undergo facile lactamization. Lactam derivatives can be found in tumours, depending on their age, storage conditions and method of extraction. Such lactams may in many cases be artefacts. Nevertheless glutamyl opine type *Agrobacterium* strains must frequently encounter the lactams as well as the glutamyl opines. It is not unreasonable that glutamyl opine type strains should have acquired catabolizing enzymes for the lactams as well as for the glutamyl opines. In all previous cases investigated the opine lactam is also metabolized by the strains utilizing the corresponding glutamyl opine: nopaline–nopaline lactam [30], agropine (+mannopine, mannopinic acid)–agropinic acid [16, 29], and D,L-succinamopine–succinopine lactam [26]. Catabolism of ornaline lactam and leucinopine lactam has not been studied.

The data of Table 1 show that six strains are capable of growth on L,L-succinamopine, but not one is able to utilize L,L-succinopine lactam, despite the fact that lactam formation is easily detectable during the isolation of L,L-succinamopine. One possibility is that opine X [25], a possible substrate for the L,L-opine utilizing enzymes in A518, A519 and A532, is not a substituted L-glutamyl opine, but rather a different *N*-substituted L-amino acid incapable of lactamization. Thus these strains might have no use for L,L-opine lactam catabolism.

The dominant iminodiacid opine of pTi Bo542-induced tumours on several plants (tobacco, sunflower, bean) is reported to be leucinopine [27]. Integration of NMR signals of the total iminodiacid fraction of A281 alfalfa tumour shows that more than 90% of the opine is L,L-succinamopine. If leucinopine and L,L-succinamopine are produced by a single T-DNA encoded enzyme, the biochemistry of the crown gall host plant must play a role in determining the ratio of opines produced in the tumour. If separate synthases are responsible for these two L,L-opines, complex T-DNA integration events may be responsible for the observed differences in opine concentration.

Stereospecificity of enzymes catalysing chemical reactions may produce *R*- or *S*-chirality but not both. We presume the enzyme(s) responsible for biosynthesis of the two opines of L^{glu} configuration, leucinopine and L,L-succinamopine, are unlikely to have evolved from enzymes that synthesize D^{glu}-opines. This reasoning leads us to conclude that opine chemistry has evolved more than once in crown gall tumour induction. Convergent evolutionary selection of iminodiacids as opines suggests that there may be underlying constraints on the chemical forms that are selected for opines.

EXPERIMENTAL

Bacteria and fermentation. Agrobacterium tumefaciens strain AT1TF is a transformant of strain A136 containing pTi AT1. Strain A114 is a cured C58 derivative [4]. Plasmids of other strains and their sources have been previously described [25]. Fermentations were carried out in liquid medium and opine disappearance was monitored as previously described [29].

Spectroscopy. Mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029). Fast atom bombardment (FAB) was carried out with xenon and DT's matrix (dithiothreitol 'magic bullet') [31]. High resolution FAB measurements employed an alternating probe and polypropylene oxide standard [32]. Proton NMR spectra were obtained on the 90 mHz Varian EM 390. Chemical shifts are reported relative to sodium dimethylsilapentane sulphonate. Circular dichroism was measured on the Roussel-Jouan Dichrographe III in the laboratory of Dr. Charles Tanford, Duke University.

Electrophoresis. Analytical electrophoresis was performed on Whatman 3 MM paper under hydrocarbon coolant at 50 V/cm. The pH 2.8 buffer was prepared by titrating 0.1 M HCO_2H with NaOH. The pH 8.3 buffer contained 0.05 M $NaHCO_3$. The pH 10.8 buffer was prepared by titrating 0.1 M NH_4OH with HOAc. Opines and their diastereoisomers were detected by chelation of silver in the presence of mannitol (reversed $AgNO_3$ test) [25]. Mobilities (u_{pic}) are reported relative to picrate.

Chemicals. Synthetic opines were prepared by reduction of L-

or D-asparagine (Sigma) and 2-ketoglutaric acid with Na cyanoborohydride as described in ref. [26].

Isolation of L,L-succinamopine. L,L-Succinamopine was isolated as the major A281 alfalfa tumour iminodiacid in two experiments. The first isolation from uncloned tumour has been previously described [28]. L,L-Succinamopine was isolated a second time from a cloned tumour tissue culture line. Frozen A281-6 alfalfa tumour (162 g) in 200 ml 95% EtOH was homogenized for 2 min in a blender. The extract was centrifuged and 265 ml of supernatant was loaded on a 2.2 × 20 cm (70 ml) column of new Biorad AG50H+, previously cycled with NH_4OH, HCl and H_2O. Thirteen 20 ml fractions, 3.6 column vols (CV), were collected during loading of the extract. The eluent was changed to H_2O. Opines were located by electrophoresis at pH 2.8. Agropinic acid emerged during loading and tailed beyond 15 CV due to continuous formation from agropine and mannopine absorbed on the column. Elution of the iminodiacid opines leucinopine and L,L-succinamopine occurred between 3 and 18 CV. Appropriate fractions were pooled and reduced to dryness. The loading fraction CV 0–3.6 contained 1.128 g sugars and agropinic acid; CV 3.6–9.0 contained 258 mg mixed pyroglutamic acid, agropinic acid and L,L-succinamopine; CV 9–18 contained 53 mg, principally L,L-succinamopine, with some pyroglutamic acid, agropinic acid and leucinopine. The combined 311 mg eluted in CV 3.6–18 was dissolved in 5 ml H_2O and loaded on a 1.1 × 15 cm (13 ml) column of Biorad AG50H+. The column was eluted with H_2O. Agropinic acid, pyroglutamic acid and L,L-succinopine lactam, formed during work up, emerged in the loading fraction. L,L-Succinamopine (25 mg), containing a very small amount of leucinopine, emerged between 5 and 15 CV.

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