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ABSOLUTE STEREOCHEMISTRY OF LEUCINOPINE, A CROWN GALL OPINE

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

Abstract—Crown gall tumors incited by *Agrobacterium tumefaciens* strain Bo542 have been reported to synthesize a tumor-specific substance identified as *N*-(1,3-dicarboxypropyl)-leucine (leucinopine), a compound with two centers of asymmetry. We report here evidence that leucinopine is indeed a crown gall opine, in that it is specifically catabolized by *A. tumefaciens* strains carrying the tumor-inducing plasmid pTi Bo542, as well as strains carrying closely related plasmids pTi AT1 and pTi AT4. We further report catabolism of leucinopine by the succinamopine-type strains A518, A519 and A532, carrying pTi EU6, pTi AT181 and pTi T10/73, respectively. Strains lacking any virulence plasmid, as well as those carrying octopine or nopaline type Ti plasmids or mannopine type Ri plasmids, did not catabolize leucinopine. On the basis of specificity of catabolism by bacteria carrying pTi Bo542, we conclude that the stereochemistry of natural leucinopine is L-threo, i.e. L^{glu},L^{leu}. Such stereochemistry is novel in the opines known thus far: octopine, nopaline and succinamopine have D,L-stereochemistry: D^{ala},L^{arg} (octopine), D^{glu},L^{arg} (nopaline) and D^{glu},L^{asn} (succinamopine).

INTRODUCTION

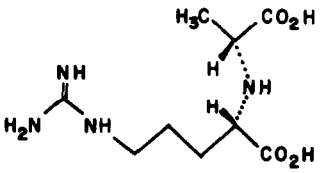
Novel metabolites are synthesized in crown gall tumors at the direction of the inciting pathogenic *Agrobacterium tumefaciens* strain [1–4]. Gene transfer from the bacterial virulence plasmid to the plant cells is responsible for these new biosynthetic activities [5–9]. The inciting bacterial strain in general can catabolize only the group of opines whose synthesis it induces in its tumors [2–4]. The crown gall tumor is thus a genetically engineered source of nutrients for the pathogen.

A new tumor-specific metabolite, *N*-(1,3-dicarboxypropyl)-leucine, leucinopine, has recently been reported in tumors incited by strain Bo542 [1]. No evidence was presented by these authors in support of the assignment of L-stereochemistry to the leucine substituent, and no stereochemical assignment was suggested for the 1,3-dicarboxypropyl substituent. Further, these authors did not investigate whether leucinopine is specifically catabolized by *A. tumefaciens* strains carrying pTi Bo542, a prerequisite for designation of leucinopine as a crown gall opine [10]. We here report the synthesis of all four diastereomers of leucinopine, only one of which is catabolized by the group of bacterial strains of which pTi Bo542 is a member. We report verification of the presence of a substance co-electrophoresing with leuinopine in tumors incited by strain A281, carrying pTi Bo542. We conclude that leucinopine fulfils the biological definition of a crown gall opine [10], and that it possesses L,L-stereochemistry, in contradistinction to the D,L-stereochemistry established for octopine [11], nopaline [12] and succinamopine [13].

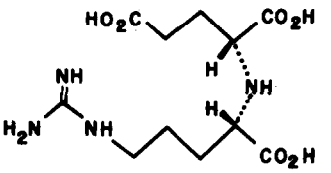
RESULTS AND DISCUSSION

L^{glu},L^{leu}- and D^{glu},L^{leu}-leucinopine diastereomers were synthesized by reductive condensation [14] of L-leucine with α-ketoglutaric acid. L^{glu},L^{leu}- and L^{glu},D^{leu}-leucinopine diastereomers were synthesized by reductive condensation of L-glutamic acid with α-ketoisocaproic acid. Similarly, D^{glu},D^{leu}- and D^{glu},L^{leu}-leucinopine diastereomers were synthesized by condensation of D-glutamic acid with α-ketoisocaproic acid. Each of the three pairs of diastereomers was subjected to fermentation by *A. tumefaciens* strain A136, which contains no Ti plasmid [15], and strain A281, which is an isogenic strain containing pTi Bo542 [15]. Each pair of diastereomers was fermented in liquid medium as described [16], and culture supernatants were examined by paper electrophoresis at pH 2.8 using silver nitrate–mannitol detection [16]. As summarized in Table 1, strain A281 catabolized only the electrophoretically more mobile diastereomer synthesized from L-leucine or from L-glutamic acid, but not from D-glutamic acid. Therefore the structure of the catabolized diastereomer must be L^{glu},L^{leu} (4). Strain A136 catabolized none of the diastereomers. As a second control, strain A208, containing the nopaline-type Ti plasmid pTi T37, was also found not to catabolize any of the diastereomers. The more mobile L^{glu},L^{leu} diastereomer synthesized from L-glutamic acid was readily separated from the less mobile diastereomer by crystallization. This pure L,L-diastereomer as expected was utilized by strain A281 but not by control strains A208 and A136. In addition, it was utilized by three succinamopine-type strains, A518, A519 and A532, carrying pTi EU6, pTi AT181 and pTi T10/73.

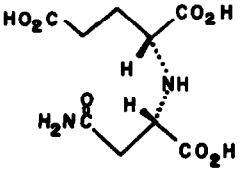
Strictly speaking, the fermentation studies only demonstrate uptake of the L,L-leucinopine diastereomer. In order to verify that the bacteria catabolize this substance, the mixed diastereomers synthesized from L-glutamic acid were used as the sole carbon source in minimal agar plates. Octopine (Sigma), synthetic nopaline [14] and synthetic mannopine [17] media were employed as controls. *Agrobacterium* strains of several types were streaked for



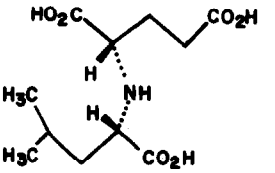
1 OCTOPINE



2 NOPALINE



3 SUCCINAMOPINE



4 LEUCINOPINE

Table 1. Utilization of synthetic leucinopine diastereomers

Strain	Virulence plasmid	Catabolism of leucinopine synthesized from: L-glu Fast	L-glu Slow	D-glu Fast	D-glu Slow	L-leu Fast	L-leu Slow
A136	None	–	–	–	–	–	–
A281	pTi Bo542	+	–	–	–	+	–
A208	pTi T37	–	–	–	–	–	–

Leucinopine diastereomeric mixtures were synthesized from L-glutamic acid (L,L- and L,D-isomers), from D-glutamic acid (D,L- and D,D-isomers) and from L-leucine (D,L- and L,L-isomers). Each diastereomeric pair was supplied to cultures of the indicated strains at 1 mg/ml in the presence of limiting mannitol as carbon source (0.05%) as described [16]. Culture supernates were assayed at intervals of 2–3 days for utilization of leucinopine. Aliquots of 30 µl were subjected to paper electrophoresis at pH 2.8 and electrophoretograms were stained with Ag NO_3/mannitol as described [16]. Diastereomers in the mixture are designated 'fast' and 'slow' based on their relative electrophoretic mobility at pH 2.8. Data presented above were obtained at 9 days after inoculation. Utilization in replicate experiments was slow, typically requiring more than 4 days.

single colonies on these media (Table 2). Growth on leucinopine was strikingly slow compared to that on other opines. The expected strains carrying agropine-type Ti plasmids, pTi Bo542, pTi AT1 and pTi AT4, all grew on leucinopine. In addition, the three succinamopine-type strains noted above, A518, and A519 and A532, grew on leucinopine. Representative octopine, nopaline and mannopine-type strains did not grow on this new opine, nor did the cured strain A136, which contains no Ti plasmid.

In order to establish that tumor tissue incited by pTi Bo542 indeed contains material corresponding to synthetic L,L-leucinopine, we examined uncloned alfalfa crown gall tissue incited by strain A281, cultured *in vitro*. An enriched iminodiacid fraction [13] contained a substance giving a reverse silver nitrate spot co-migrating with L,L-leucinopine at pH 2.8. In addition, the fraction contained agropinic acid and a further unknown (Table 3). This fraction was subjected to fermentation by strains A136, A208, A277, A281, A543 and A519. As shown in Table 3, strains A281, A543 and A519 utilized both the unknown substance and the presumptive leucinopine, while control plasmidless, octopine and nopaline strains (A136, A208 and A277, respectively) utilized

Table 2. Utilization of opines by *Agrobacterium* strains

Strain	Type	Virulence plasmid	Growth on OCT	NOP	MOP	SAP	LOP
A136	–	none	–	–	–	–	–
C58	NOP	pTi C58	–	+++++	–	–	–
O362	NOP	pTi O362	–	+++++	–	–	–
A203	NOP	pTi 223	–	+++++	–	–	–
T37 Braun	NOP	pTi T37	–	+++++	–	–	–
A178	NOP	pTi K27	–	+++++	–	–	–
A208	NOP	pTi T37	–	+++++	–	–	–
IIBV7	NOP	pTi IIBV7	–	+++++	–	–	–
A277	OCT	pTi B6-806	+++++	–	+++++	–	–
15955	OCT	pTi 15955	+++++	–	+++++	n.t.	–
A136 (pTi A6 Binns)	OCT	pTi A6 Binns	++++	–	+++++	n.t.	–
R10	OCT	pTi R10	++++	–	+++++	–	–
Ach5	OCT	pTi Ach5	++++	–	+++++	–	–
B6-806	OCT	pTi B6-806	+++++	–	+++++	–	–
A518	SAP	pTi EU6	–	–	–	+++++	+++
A519	SAP	pTi AT181	–	–	–	+++++	+++
A532	SAP	pTi T10/73	–	–	–	+++++	+++
542TF3	AGR	pTi Bo542	–	–	++++	n.t.	++
542TC2	AGR	pTi Bo542	–	–	++++	n.t.	+
AT1TF	AGR	pTi AT1	–	+	+++++	n.t.	++
A281	AGR	pTi Bo542	–	+	+++++	–	+
A543	AGR	pTi AT4	–	–	+++	–	++
TR7TF	MOP	pRi TR7	–	–	+++++	n.t.	–
TR101	MOP	pRi TR101	–	–	+++++	n.t.	–

Sources of the strains employed have been described [10, 15–17]. Bacteria were streaked for single colonies on minimal agar [17] containing synthetic opine as the sole carbon source at 1–2 mg/ml. Octopine was purchased from Sigma; other opines were synthesized by the authors. The leucinopine used for this study was prepared from L-leucine. Growth was scored subjectively as strong (+++++) to weak (+) based on colony size at 10 days. Growth on leucinopine was slowest, and was not detectable at 3–4 days when other plates were fully grown. Succinamopine (SAP) catabolism data were taken from a separate study [16]. N.T. = not tested.

Abbreviations: OCT, octopine; NOP, nopaline; MOP, mannopine; SAP, succinamopine; LOP, leucinopine; AGR, agropine.

Table 3. Utilization of tumor extract by *Agrobacterium* strains

Strain	Utilization of: AGA	UNK	LOP
A136	–	–	–
A208	–	–	–
A277	+	–	–
A281	+	+	+
A543	+	+	+
A519	–	+	+

The iminodiacid fraction from an alfalfa tumor incited by strain A281 was purified as described above and subjected to fermentation analysis as described for Table 1. Abbreviations: AGA, agropinic acid; UNK, an unknown substance (see text); LOP, leucinopine. Strains are described in Table 2.

neither. The expected strains utilized the agropinic acid in this mixture (A277, A281, and A543). These results confirm the utilization of natural leucinopine by the bacterial strains found to catabolize synthetic leucinopine.

The very slow utilization of leucinopine may be due to toxicity of its catabolic breakdown products. By analogy with the catabolic pathways for other opines, leucinopine might be oxidized to L-leucine plus α-ketoglutaric acid, or to L-glutamic acid plus α-ketoisocaproic acid. None of the succinamopine-type or agropine-type strains grows on L-leucine as the sole carbon source, while all of these strains, as well as plasmidless strain A136, grow moderately on L-glutamic acid as the sole carbon source.

The unknown substance noted in Table 3 appears to be an opine of A281 tumor tissue. It was fractionated by a method that selects for iminodiacids, and it is utilized by the same type strains as is leucinopine. We speculate that this new opine may be a second substance with the novel L,L-stereochemistry proven here for leucinopine. The extremely poor growth of the leucinopine-catabolizing strains on leucinopine as sole carbon source is surprising in light of the hypothesis that opines have a nutritionally

beneficial role for the *Agrobacterium* strains that incite crown gall tumors. It is possible that the unknown opine may be a substance with more useful nutritional characteristics, and that leucinopine may be a minor opine formed as a by-product due to low substrate specificity on the part of the synthase.

EXPERIMENTAL

Electrophoretic analysis. Electrophoreses were carried out at pH 2.8 on Whatman 3 MM paper at 50 V/cm under hydrocarbon coolant. The buffer was prepared by titrating 0.1 M formic acid with NaOH. The leucinopines were detected by $AgNO_3$ chelation [16]. Mobilities (u_{pic}) are reported relative to picrate anion.

L^{leu},L^{glu}- and L^{leu},D^{glu}-leucinopine. Mixed diastereomeric leucinopines were prepared by standard methods [1, 14] from 8.54 g (58.6 mmol) α-ketoglutaric acid, 4.81 g (36.7 mmol) L-leucine and 2.55 g (63 mmol) Na borocyanohydride. The product was absorbed onto 320 ml Dowex 50. The resin was washed with 500 ml H_2O, followed by 2 M NH_4OH. A white ppt. of leucinopine formed on the column just ahead of the advancing NH_4OH front, but redissolved as the ammonia passed the precipitation zone. The major leucinopine-containing fractions, identified by electrophoresis at pH 2.8, were pooled and concd to 50 ml, from which 0.97 g leucine crystallized. Further concn of the mother liquor gave 2.50 g mixed leucinopine diastereomers (pH 2.8, u_{pic} 0.47 and 0.57).

L^{leu},L^{glu}- and D^{leu},L^{glu}-leucinopines. The mixed leucinopine diastereomers were prepared from 1.69 g (10 mmol) Na L-glutamate, 4.56 g (30 mmol) Na α-ketoisocaproate and 1.18 g (31 mmol) Na borocyanohydride. The reaction mixture was absorbed on 100 ml Dowex 50, which was washed with 300 ml H_2O. The resin was eluted with 2 M NH_4OH which caused transient precipitation of leucinopine on the column. Evapn of the aq. NH_4OH gave 2.15 g mixed leucinopine diastereomers (pH 2.8, u_{pic} 0.50 and 0.58).

L^{leu},L^{glu}-Leucinopine (**4**). A portion of the diastereomeric leucinopines prepared from L-glutamic acid was fractionally crystallized from H_2O, giving a first crop of 112 mg of the higher mobility leucinopine diastereomer (L,L). Chilling the mother liquor gave a second crop of 433 mg of the higher mobility diastereomer. The soluble residue, 903 mg recovered as crystals in an amorphous matrix, was predominantly the lower mobility diastereomer (D,L) but contained a detectable amount of the more mobile diastereomer and a small amount of leucine detectable with ninhydrin.

L^{leu},D^{glu}- and D^{leu},D^{glu}-leucinopines. Leucinopines were prepared from 0.59 g (4.0 mmol) D-glutamic acid (Sigma), 1.86 g (12.2 mmol) α-ketoisocaproic acid and 0.43 g (7.0 mmol) Na borocyanohydride. The product was absorbed onto 60 ml Dowex 50 and eluted with 2 M NH_4OH. Pooled leucinopine fractions were evapd to dryness to avoid fractionation of diastereomers by crystallization. Electrophoretic analysis at pH 2.8 showed the presence of the two leucinopine diastereomers (u_{pic} 0.50 and 0.58) and a trace of glutamic acid detectable with ninhydrin.

Isolation of tumor leucinopine. Uncloned alfalfa tumor incited by *A. tumefaciens* strain A281 was cultured on Murashige–Skoog medium [18]. Frozen tumor (158 g) was thawed and ground in 200 ml EtOH. The supernatant from centrifugation was loaded on 60 ml of new Dowex 50 resin which had previously been put through a cycle of NH_4OH and HCl washing. Fractions were collected while the column was washed with 1.2 l. H_2O followed by 2 M NH_4OH. The first 200 ml, containing reducing sugars, was discarded. The following 660 ml contained 110 mg solid, consisting of comparable amounts of agropinic acid (pH 2.8, μ_{pic} 0.35), an unknown which chelates $AgNO_3$ (pH 2.8, u_{pic} 0.50) and leucinopine co-migrating with the more mobile synthetic leucinopine diastereomer (pH 2.8, μ_{pic} 0.58). The succeeding 640 ml H_2O eluate contained 75 mg solid, mostly agropinic acid with a trace of the other two compounds. The 400 ml NH_4OH breakthrough fraction contained 658 mg solid consisting of the standard amino acids plus mannopine and mannopinic acid.

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NOTE ADDED IN PROOF

Succinamopine type *A. tumefaciens* strains A518, A519 and A532 catabolize the *erythro* as well as the *threo* isomer of leucinopine synthesized from L-leucine.