the uncommon *R* configuration (Biellmann et al., 1977; Biemann et al., 1960).

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Registry No. **1**, 71-00-1; **2a**, 62504-27-2; **2b**, 85027-27-6; **3**·HCl, 90269-13-9; L-histidine hydrochloride, 645-35-2; (*S*)-2-bromopropionic acid, 32644-15-8; L-alanine, 56-41-7; (±)-2-bromopropionic acid, 10327-08-9; 1-benzyl-L-histidine, 16832-24-9; imidazole, 288-32-4; 2-bromopropionic acid, 598-72-1; pyruvic acid, 127-17-3; (*R*)-2-bromopropionic acid, 10009-70-8.

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Structure and Stereochemistry of Succinamopine†

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ABSTRACT: *Agrobacterium tumefaciens* strains A518, A519, AT181, and EU6 incite crown gall tumors on tobacco that produce a new opine, succinamopine, not found in healthy tobacco. NMR, mass spectral analysis, synthesis, selective fermentation of diastereomers, and circular dichroism show that succinamopine is *N*-[(1*S*)-1-carboxy-2-carbamoylethyl]-(*R*)-glutamic acid (**1a**), formally the reductive conjugate of (*S*)-asparagine and α-ketoglutaric acid. Succinamopine is rapidly converted in acid into succinamopine lactam (**2a**) and finally into succinopine lactam (**2b**). Succinamopine and the two lactams are catabolized selectively by *A. tumefaciens* strains A518, A519, and A532 but not by the isogenic strain A136, which lacks the virulence plasmid.

The organism *Agrobacterium tumefaciens*, the causitive agent of crown gall in dicotyledonous plants, harbors a tumor-inducing (Ti) plasmid. A portion of the Ti plasmid (T DNA) is incorporated into the host plant genome and causes plant cells containing T DNA to proliferate as a cancerous growth independent of normal hormonal regulation. The T DNA contains a gene, expressed in the plant, that is responsible for production of one or more unusual metabolites called opines (Guyon et al., 1980). Most opines so far identified appear to be reductive condensation products of an L-amino acid (lysine, arginine, leucine, glutamine) and a carbonyl compound (pyruvic acid, α-ketoglutaric acid, mannose) (Biemann et al., 1960; Menagé & Morel, 1964; Goldmann et al., 1969; Firmin & Fenwick, 1978; Tate et al., 1982; Chang et al., 1983). The Ti plasmid also contains a gene, not a part of T DNA, expressed in the bacterium that permits the pathogenic bacterium to catabolize the particular opine that its plasmid causes the plant tumor to synthesize (Petit et al., 1970). Ti plasmids have been characterized by biochemical

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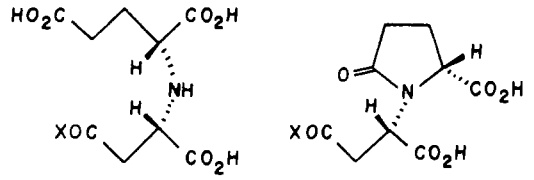
type according to the opine that the bacterial Ti plasmid can catabolize (Sciaky et al., 1978). An *A. tumefaciens* strain containing a Ti plasmid can thrive on its opine as the sole carbon source. In addition, some strains have the ability to catabolize structurally related opines from other strains. Several such strains that catabolize nopaline (**3**) poorly, but do not cause tumors producing nopaline, have been recently shown to cause tumors producing another opine, succinamopine (Chilton et al., 1984). In this paper, we detail the physical, spectroscopic, chemical, and fermentation properties of succinamopine (SAP) (**1a**) and its facile transformation products succinamopine lactam (SAL) (**2a**) and succinopine lactam (SOL) (**2b**), which prove their structure and absolute stereochemistry.



1a X = NH_2 **2a** X = NH_2

1b X = OH **2b** X = OH

Materials and Methods

Spectroscopy. Nuclear magnetic resonance spectra were measured on a JEOLCO 60-MHz spectrometer, infrared was measured in KBr pellets on a Perkin-Elmer IR 5B spectrometer, and circular dichroism was measured on a JASCO Durrum J20 spectrometer.

High-resolution and fast atom bombardment (FAB) 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). High-resolution masses were calibrated with perfluorokerosene and FAB masses with glycerol cluster ions.

Electrophoresis. Electrophoresis was conducted on Whatman 3MM paper under hydrocarbon (Stoddard) coolant at 50 V/cm. Adequate resolution was generally achieved in 30 min or less. Mobilities (u_{pic}) are reported relative to picrate. The alternative mobility standard Orange G II had a u_{pic} 1.25 ± 0.06 over the pH range 1.8–6.1. The pH 1.8 buffer was prepared by titrating 2 M acetic acid with formic acid to pH 1.8. Buffers in the range pH 2.4–3.8 were prepared by titrating 0.1 M formic acid with concentrated KOH. Buffers in the range pH 3.6–5.9 were prepared by titrating 0.1 M acetic acid with KOH. Buffers for the range pH 3.7–6.3 were also prepared with 0.05 M succinic acid. Buffers in the range pH 9.8–10.6 were prepared by titrating 0.1 M ammonia with acetic acid. Relative mobilities of the opines were dependent only on the pH and not on the identity of the buffer anion. Relative mobilities were unaffected by changing the cation to sodium.

Chemicals. Nopaline was isolated from tobacco tumors as previously described (Goldmann et al., 1969). Nopaline lactam was prepared by allowing an acidified solution of nopaline to stand for 5 days. Concentration gave crystalline nopaline lactam, which was recrystallized from water: mp 255 °C dec; FAB quasi molecular ion M + 1 at 287. Anal. Calcd for $C_{11}H_{18}N_4O_5$: C, 46.13; H, 6.34; N, 19.58. Found: C, 46.12; H 6.84; N 19.57. Agropinic acid was prepared via mannopine (Tate et al., 1982). Other chemicals were purchased from Sigma Chemical Co.

Tissue Culture and Bacterial Fermentation. A cloned crown gall tumor line, TA 519-8, induced by *A. tumefaciens* strain A519 on Havana tobacco was grown on large scale for preparative production of succinamopine as previously described (Chilton et al., 1984).

Fermentation of synthetic succinamopine and succinopine lactam diastereomers by succinamopine-type *A. tumefaciens* strains A518, A519, and A532 was carried out in Bergersen's medium (Bergersen, 1961) supplemented with 0.1% ammonium sulfate, 0.4 mg/mL mannitol, and 1 mg/mL synthetic opine, adjusted to pH 7.0 and filter sterilized. Successful fermentation led to complete removal of the opine from the medium within 72 h as determined by electrophoresis at pH 2.8. Fermentation of the natural opine under the same conditions and nonfermentation by the isogenic strain A136, lacking the Ti plasmid, were used as controls.

Fermentability of synthetic opines was also assessed by observation of growth (large, white colonies) or nongrowth of bacterial strains A518, A519, and A532 on 0.7% agar containing 1 mg/mL synthetic opine as the sole carbon source in AT medium (Guyon et al., 1980) supplemented with 0.2% ammonium sulfate. Lack of growth by the isogenic strain A136 was used as a negative control.

Chemical Detection of New Opines. Succinamopine and related opines may be detected by bromcresol green (Zweig & Sherma, 1972), by chelation of silver in the presence of mannitol (reversed silver nitrate test) (Chilton et al., 1984), or by ferric ion chelation (ferric thiocyanate) (Firmin & Gray, 1974). The detection limit for succinamopine and succinamopine lactam with silver nitrate and mannitol after electrophoresis at pH 2.8 is below 5 μg. This test is less sensitive after electrophoresis in the other buffers described and is considerably less sensitive for succinopine lactam (limit 20 μg). The detection sensitivity for succinopine lactam is slightly better with bromcresol green.

Succinamopine and succinamopine lactam may also be detected by hypochlorite, followed by benzidine or tolidine (Chilton et al., 1984), but carcinogenicity of the redox-indicator amines should be kept in mind. Other diagnostic tests used in preliminary testing of the new opine for the possible presence of functional groups (dinitrophenylhydrazine test for ketones, quinoxalinol test for α-keto acids, and ferric hydroxamate test for esters) are described by Zweig & Sherma (1972).

Isolation of Succinamopine (**1a**). Frozen tumor (850 g) was ground with 1150 mL of 95% ethanol. The extract was centrifuged, filtered, and passed through a 2.2 × 30 cm column of Dowex 50 H^+ (50–100 mesh) at 120 mL/h. The column was then immediately developed with distilled water. Analytical electrophoresis at pH 2.8 was used to locate fractions containing succinamopine and glutamic acid lactam. The elution of these acidic compounds could also be followed by plotting the pH of the fractions. A background of glutamic acid lactam bled off the column continuously as long as there was any glutamic acid still bound ionically to the Dowex 50. Succinamopine emerged between 0.2 and 0.9 L and was contaminated by glutamic acid lactam. The succinamopine-containing fractions were pooled and concentrated to 0.5 g of a thick oil, which was dissolved in 3 mL of water and loaded on a 1.1 × 20 cm column of Dowex 50 H^+. Distilled water eluted first glutamic acid lactam and then succinamopine in discrete peaks detected by plotting pH vs. fraction number. A small amount of opine X (Chilton et al., 1984), a homologue of succinamopine, eluted in late fractions of succinamopine. Pooled succinamopine fractions were lyophilized to a white

Table I: Fermentation of Succinopine Lactam Diastereomeric Pairs

reactants			succinopine lactam		pH 2.8	fermented
chiral compound	achiral compound	method	Asp center	Glu center	mobility	by A519
(*S*)-Asn	α-KG	reduction	*S*	*R*	0.98	+
			S	*S*	0.66	−
(*R*)-Asp	α-KG	reduction	*R*	*S*	0.98	−
			R	*R*	0.66	−
(*R*)-Glu	OAA	reduction	*R*	*R*	0.66	−
			S	*R*	0.98	+
(*S*)-Glu	(*RS*)-bromosuccinic acid	alkylation	*R*	*S*	0.98	−
			S	*S*	0.66	−

powder. Electrophoresis of this preparation at pH 2.8 and development with silver nitrate–mannitol or with hypochlorite–tolidine showed it to contain succinamopine, a trace of opine X, and no glutamic acid lactam. Electrophoretic mobility data for succinamopine are given in Figure 1.

Conversion to Succinamopine Lactam (**2a**). Succinamopine isolated by double elution from Dowex 50 as described above was allowed to stand in the elution water (pH 2) for several days. The progress of lactamization was monitored electrophoretically at pH 2.8. When lactamization was nearly complete, the aqueous solution was concentrated to 3 mL and passed through a 1.1 × 20 cm column of Dowex 50 H^+ to remove any residual succinamopine. Succinamopine lactam, eluting immediately after the holdup volume, was recovered by lyophilization. Purity was checked electrophoretically at pH 2.8 by detection with silver nitrate–mannitol, bromcresol green, and hypochlorite–tolidine. Electrophoretic mobility data are given in Figure 1.

Conversion to Succinopine Lactam (**2b**). A 977-mg mixture of succinamopine and glutamic acid lactam, isolated by a single elution from Dowex 50, was dissolved in 20 mL of 1 M HCl and heated at reflux for 90 min, cooled, and evaporated to 969 mg of oil. The crude succinopine lactam was dissolved in 20 mL of water and loaded on a 2.2 × 18 cm column of Dowex 1 formate. The column was developed at 80 mL/h with an exponential formic acid gradient formed by adding 6 M formic acid to distilled water in a 500-mL constant-volume mixing chamber. Succinopine lactam emerged after the gradient reached 4.5 M formic acid, well resolved from accompanying glutamic acid lactam, which was eluted at ca. 0.2 M formic acid. Succinopine lactam (359 mg) was recovered by evaporation of pooled fractions. Electrophoretic analysis of succinopine lactam at pH 1.8, 2.8, and 10.0 with detection by silver nitrate–mannitol and bromcresol green showed it to be free of glutamic acid lactam and the other opines. Electrophoretic mobility data are given in Figure 1 and Table I.

Synthesis of Diastereomeric Succinopine Lactams. A solution of 23.4 g (0.16 mol) of α-ketoglutaric acid, 21.3 g (0.16 mol) of (*S*)-aspartic acid, and 17.3 g of NaOH was adjusted to pH 6 by addition of 2 M NaOH to give a total volume of 270 mL. Portions of 10.8 g (0.18 mol) of sodium cyanoborohydride were added over a period of 4 h, and the pH was periodically readjusted to the range 6–7 by addition of small portions of powdered α-ketoglutaric acid. The reaction solution was allowed to stand overnight and then poured into a beaker containing 930 mL of Dowex 50 H^+ in a fume hood (HCN evolution). The slurry was stirred vigorously for 10 min, during which gas evolution was noted. The aqueous solution was filtered from the resin, and the resin was washed with additional water to give a total filtrate of 1 L of clear, aqueous bubbling solution. The resin was resuspended in 800 mL of water, stirred 5 min, and refiltered. The second filtrate showed no gas evolution, while the first filtrate continued to bubble. Evaporation of the second filtrate gave 3 g of white solid, containing succinopine (**1b** and diastereomer).

The resin, which retained most of the succinopine, was transferred to a 2-L flask with a little deionized water. The resin slurry was adjusted to pH below 1 by addition of HCl and was heated at reflux for 90 min. The cooled resin slurry was poured into a 4.7 × 54 cm column and washed with deionized water until the acidity of the effluent reached pH 4. The total eluate (3 L) was concentrated to 17.6 g of a slightly yellow oil, which partially crystallized on standing. Electrophoretic analysis at pH 2.8 showed the presence of a pair of succinopine lactam diastereomers at a u_{pic} 0.69 and 0.98, detectable with bromcresol green or with silver nitrate–mannitol.

Separation of Synthetic Succinopine Lactam Diastereomers. A solution of 15 g of diastereomeric succinopine lactams in 100 mL of water was loaded on a 4.7 × 48 cm column of Dowex 1 acetate. The column was developed with a three-step exponential gradient created in a 1-L constant-volume mixing chamber filled initially with distilled water. Formic acid was fed to the mixing chamber in three steps: 3 L of 4 M formic acid, followed by 1.5 L of 5 M, and then 1.5 L of 6 M formic acid. Fractions were analyzed by electrophoresis at pH 2.8 with silver nitrate–mannitol detection. Elution of ($S^{Asp}S^{Glu}$)-isosuccinopine[1] lactam began at about 3.5 M formic acid, while elution of the ($S^{Asp}R^{Glu}$)-succinopine lactam began at 4.8 M formic acid, with some overlapping fractions. Evaporation of appropriate pure fractions gave 3.0 g of ($S^{Asp}S^{Glu}$)-isosuccinopine lactam [*N*-[(1*S*)-1,2-dicarboxyethyl]-(5*S*)-2-pyrrolidone-5-carboxylic acid]. The NMR of the (*SS*)-lactam in D_2O at pD 1 had proton signals at 2.50 (m, glutamyl β,γ-H), 2.71 (AB of ABX, aspartyl β,β′-H), 4.45 (m, glutamyl α-H), and 4.77 ppm (t, J = 6 Hz, aspartyl α-H). Evaporation of pure ($S^{Asp}R^{Glu}$)-succinopine lactam fractions gave 5.0 g of *N*-[(1*S*)-1,2-dicarboxyethyl]-(5*R*)-2-pyrrolidone-5-carboxylic acid (**2b**). The NMR of synthetic (*SR*)-lactam was identical with that of succinopine lactam isolated from tobacco tumor.

The separated diastereomers give identical mass spectra but are readily distinguishable by electrophoretic mobility (Table I), circular dichroism (Table II), thin-layer chromatography (TLC), and NMR. The (*SS*)-isosuccinopine lactam has an R_f of 0.25 on silica gel TLC developed with 1-butanol–acetic acid–water (12:3:5); (*SR*)-succinopine lactam has an R_f of 0.18. The 60-MHz NMR spectrum of (*SR*)-succinopine lactam in D_2O at pD 1 has an isochronous aspartyl β,β′-H signal (d, coupled with the aspartyl α-H), while the β,β′-H signal of (*SS*)-isosuccinopine lactam is an anisochronous ABX

[1] Diastereomers prepared in this study that have erythro relative stereochemistry are referred to as succinopine and succinamopine; those having threo stereochemistry are referred to as isosuccinopine and isosuccinamopine. For brevity, the chirality of the aspartyl and the glutamyl α-carbons in the succinamopine family of opines is referred to as R^{Asp}, S^{Asp}, R^{Glu}, or S^{Glu} as appropriate.

multiplet at pD 1. The NMR spectra of succinopine lactam trimethyl ester and its diastereomer in carbon tetrachloride have two signals in the ratio 1:2 for the three methoxyl groups with the larger signal upfield of the smaller in the spectrum of (*SS*)-isosuccinopine lactam trimethyl ester and reversed in (*SR*)-succinopine lactam trimethyl ester.

Synthesis of Further Diastereomeric Pairs. (A) ($R^{Asp}R^{Glu}$)- *and* ($S^{Asp}R^{Glu}$)-*Lactams from* (*R*)-*Glutamic Acid.* A solution of 645 mg (4.38 mmol) of (*R*)-glutamic acid in 10 mL of 2 M NaOH was neutralized to pH 6.8 by addition of 1.30 g of oxaloacetic acid (effervescence), and 374 mg of sodium cyanoborohydride was added. The pH was monitored and kept in the range 6–7 by addition of small portions of oxaloacetic acid. A total of 1.94 g (17.3 mmol) of oxaloacetic acid was used. The solution was allowed to stand 1 day and was then poured onto 50 mL of stirred Dowex 50 H^+ in the fume hood. The resin was removed by filtration and washed with deionized water. The drained resin was transferred with a little water to a flask, 5 mL of 1 M HCl was added, and the resin slurry was heated at reflux for 2 h. The resin slurry was loaded in a column and washed with 500 mL of deionized water. Evaporation of the eluate gave 837 mg of partially crystalline material, containing by electrophoresis at pH 2.8 the expected diastereometric ($R^{Asp}R^{Glu}$)-isosuccinopine lactam *N*-[(1*R*)-1,2-dicarboxyethyl]-(5*R*)-2-pyrrolidone-5-carboxylic acid and the ($S^{Asp}R^{Glu}$)-succinopine lactam *N*-[(1*S*)-1,2-dicarboxyethyl]-(5*R*)-2-pyrrolidone-5-carboxylic acid (**2b**). In addition the preparation also contains the diastereomeric pair of propanopine lactams [*N*-(1-carboxymethyl)-(5*R*)-2-pyrrolidone-5-carboxylic acids] arising from reductive alkylation of (*R*)-glutamic acid with pyruvic acid derived from decarboxylation of oxaloacetic acid.

(B) Resolution of (*RR*)- *and* (*SR*)-*Lactams.* Resolution was obtained by elution of 600 mg of the mixed lactams from a 1.1 × 35 cm column of Dowex 1 formate with an exponential formic acid gradient formed by addition of 4.5 M formic acid to a 250-mL constant-volume mixing chamber. Fractions containing lactams were located by analytical electrophoresis at pH 2.8. Pooled ($S^{Asp}R^{Glu}$)-succinopine lactam fractions gave 71 mg of *N*-[(1*S*)-1,2-dicarboxyethyl]-(5*R*)-2-pyrrolidone-5-carboxylic acid (**2b**) of good electrophoretic purity, but the 400 mg from the pooled ($R^{Asp}R^{Glu}$)-isosuccinopine lactam fractions contained substantial *N*-(1-carboxyethyl)-2-pyrrolidone-5-carboxylic acid.

(C) Mixed ($S^{Asp}S^{Glu}$)- *and* ($R^{Asp}S^{Glu}$)-*Lactams from* (*S*)-*Glutamic Acid.* A solution of 1.6 g (10.7 mmol) of (*S*)-glutamic acid in 10 mL of 2 M KOH was treated with a drop of phenolphthalein (red color), and 2.09 g (10.6 mmol) of (*RS*)-bromosuccinic acid was added, discharging the red color. KOH was added to restore a pink color. Further KOH was added periodically as the pink color was discharged by liberated HBr. Electrophoretic analysis of the reaction solution the next day showed the presence of succinopine diastereomers and malic acid. The reaction solution was loaded on a 2.2 × 22 cm column of Dowex 50 H^+. The column was washed with 50 mL of deionized water, followed by 1 M ammonia. Breakthrough of ammonia was detected by coelution of phenolphthalein in its red form. The malic acid was washed out in early fractions. Succinopine immediately preceded and followed ammonia breakthrough. Succinopine fractions were combined and evaporated to give 823 mg of diastereomeric succinopines.

The succinopines were converted into their lactams by dissolving 576 mg in 10 mL of 0.5 M HCl and heating at reflux for 90 min. Electrophoresis at pH 2.8 showed that this preparation contained only the expected diastereomeric lactams.

(D) Mixed ($R^{Asp}S^{Glu}$)- *and* ($R^{Asp}R^{Glu}$)-*Lactams from* (*R*)-*Aspartic Acid.* The pH of a solution of 3.65 g (25 mmol) of α-ketoglutaric acid and 2.60 g (19.5 mmol) of (*R*)-aspartic acid in 35 mL of 2 M NaOH was adjusted to pH 6.6 by addition of solid α-ketoglutaric acid. A total of 1.76 g (27 mmol) of sodium cyanoborohydride was added in portions over the course of 3 h, with periodic addition of small portions of α-ketoglutaric acid to adjust to pH 6–7. The next day, the reaction solution was added to 210 mL of Dowex 50 H^+ in a fume hood and filtered after 5 min. The resin was resuspended by addition of 100 mL of water and refiltered. The resin was poured into a 4.7 × 13 cm column and eluted with 1 M ammonia. Fractions were analyzed by electrophoresis at pH 2.8. Succinopine and aspartic acid eluted around the ammonia breakthrough point. Pooled succinopine fractions, containing aspartic acid, were concentrated to an oil. The oil was dissolved in 30 mL of 1 M HCl and heated at reflux for 1 h. Evaporation of the aqueous HCl left 4.33 g of a crystalline solid adhering to the flask walls. To remove aspartic acid, a 3.77-g portion of the solid was dissolved in 10 mL of water, absorbed onto a 2.2 × 18 cm column of Dowex 50 H^+, and eluted with water. Evaporation of the first three column volumes gave 1.1 g of the mixed ($R^{Asp}S^{Glu}$)-lactam *N*-[(1*R*)-1,2-dicarboxyethyl]-(5*S*)-2-pyrrolidone-5-carboxylic acid and the ($R^{Asp}R^{Glu}$)-isosuccinopine lactam *N*-[(1*R*)-1,2-dicarboxyethyl]-(5*R*)-2-pyrrolidone-5-carboxylic acid, free of aspartic acid.

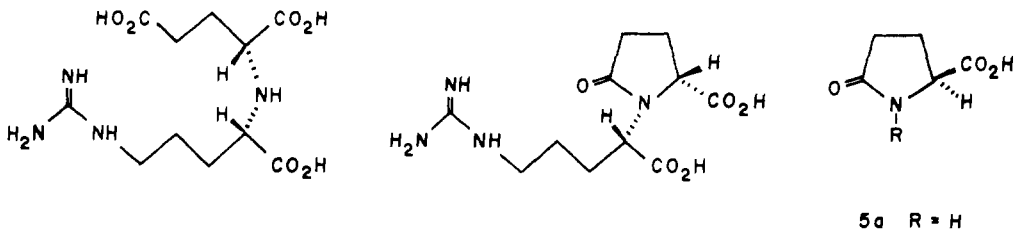
(E) Esterification. Methyl and ethyl esters were prepared by dissolving either succinopine lactam or isosuccinopine lactam in 0.5 M ethanolic or methanolic HCl and allowing the solution to stand 1 day. The alcoholic HCl was then removed at reduced pressure and the residue taken up in carbon tetrachloride and extracted with aqueous sodium bicarbonate followed by water. Esters were recovered by evaporation of the carbon tetrachloride.

The NMR spectrum of succinopine lactam trimethyl ester in carbon tetrachloride had signals of appropriate areas at 2.20 (m, glutamyl β,γ-H), 2.80 (d, J = 6 Hz, aspartyl β,β′-H), 3.54 (s, 1 methoxy), 3.60 (s, 2 methoxys), 4.30 (m, glutamyl α-H), and 4.85 ppm (t, J = 6 Hz, aspartyl α-H). Isosuccinopine lactam trimethyl ester in carbon tetrachloride had signals at 2.32 (m, glutamyl β,γ-H), 2.77 and 2.97 (d, J = 6 Hz, aspartyl β,β′-H), 3.60 (s, 2 methoxys), 3.63 (s, 1 methoxy), 4.22 (m, glutamyl α-H), and 4.53 ppm (t, J = 6 Hz, aspartyl α-H).

Results

Characterization of New Opines. Succinamopine, shown to have structure **1a**, is readily transformed in dilute acid into succinamopine lactam (**2a**). Facile lactamization is a characteristic of the glutamyl opines nopaline, agropine (Tate et al., 1982), and leucinopine (Chang et al., 1983). Lactamization is complete at pH 2 in a few days. At elevated temperature both succinamopine and succinamopine lactam are converted into a third compound, succinopine lactam, shown to have structure **2b**, with liberation of ammonia, detectable with Nessler's reagent. The pH titration and electrophoretic mobility profiles of all three opines (Figure 1) indicate that they have several acidic groups with merged ionization steps in a pH range characteristic of carboxylic acids. The molecular weights and first ionization constants of succinamopine lactam and succinopine lactam are sufficiently close that they are not resolved below pH 3. Above pH 3, the difference in their second ionization constants permits their electrophoretic resolution. Above pH 4, a third acidic group in succinopine

Chart I



3 4 5a R = H 5b R = 1-deoxy-D-mannityl

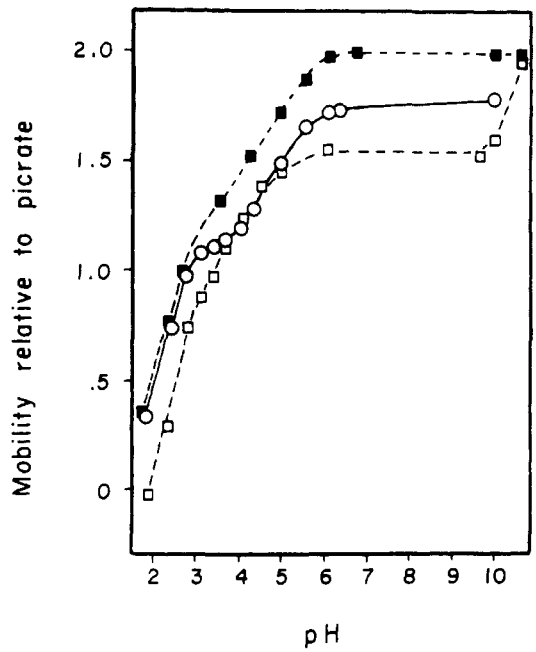


FIGURE 1: Electrophoretic mobility vs. pH. Mobility of succinamopine (□), succinamopine lactam (○), and succinopine lactam (■) in sodium and potassium formate, acetate, and succinate buffers and in ammonium acetate buffers.

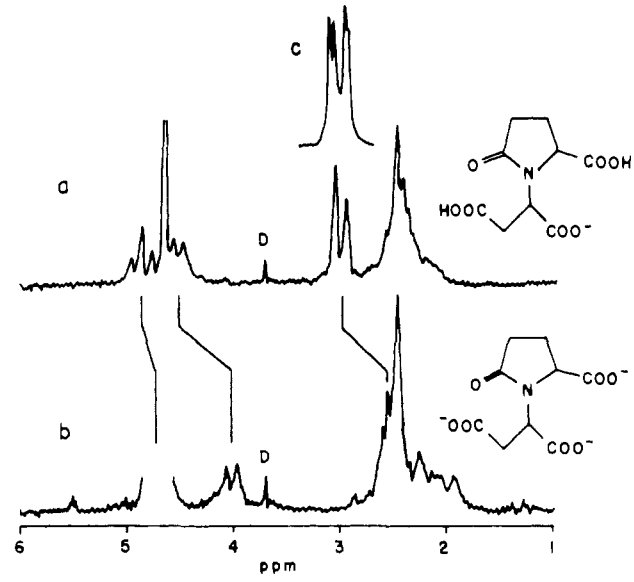


FIGURE 2: Proton NMR spectra of succinopine lactam derived from tobacco tumor incited by *A. tumefaciens* strain A519: (a) solvent D_2O, pD 2, D indicates dioxane standard; (b) solvent D_2O, pD 7, D indicates dioxane standard; (c) aspartyl methylene signal of succinopine lactam trimethyl ester in CCl_4.

lactam begins to ionize. This third group is masked as an amide in succinamopine lactam; consequently, these two compounds remain resolved electrophoretically in the alkaline pH region. The two ionization steps of succinamopine lactam are barely resolved in the pH–mobility curve and indicate first and second dissociation constants of about 2.3 and 4.7.

The first pK_a in each of the new opines is very low ($pK_a < 2.4$), indicative of a strongly electron-withdrawing α-substituent such as α-amino, α-acylamino, α-keto, and α-(acyloxy) groups. None of the three opines gives positive tests for α-keto group (dinitrophenylhydrazine or quinoxalinol) or for ester (ferric hydroxamate), but succinamopine and succinamopine lactam give a positive hypochlorite–tolidine test for the presence of amide or amine NH. Although succinamopine gives no ninhydrin color reaction within 30 min at 80 °C (absence of primary amine), it moves very slightly in the cationic direction in pH 1.8 electrophoresis and is retained from 50% ethanol on a cation-exchange resin. These facts suggest the presence of the imino diacid group found in octopine and nopaline (3; see Chart I), and the facile lactamization strongly points to a glutamyl residue in the imino polyacid succinamopine.

The first pK_a of octopine is 1.4 (Izumiya et al., 1957) and that of other imino diacids is below 2 (Schwarzenbach et al., 1949). Thus, at pH 1.8, simple imino diacids are fractional cations, as observed for succinamopine. The isoelectric point of succinamopine is about pH 1.9. Unless the pH is maintaned considerably below 1.9, water alone is sufficient to elute succinamopine from a cation-exchange resin without significant elution of aspartic acid (p*I* of 2.77), glutamic acid (p*I* of 3.22), or neutral amino acids (p*I* of 6.1). The succinamopine isolation procedure is based on this unique elution by distilled water.

The first acid transformation product succinamopine lactam lacks the basic nitrogen of succinamopine as indicated by its coelution with glutamic acid lactam from Dowex 50, immediately after the void volume, and by the failure to observe cationic electrophoretic migration of succinamopine lactam at pH 1.7. Tertiary amides and lactams lacking the amide NH are not detectable with hypochlorite tolidine; however, succinamopine lactam gives a positive test with tolidine hypochlorite, indicating the presence of a second, nontertiary amide. The presence of a second nitrogen in succinamopine lactam is confirmed by mass spectrometry. Since ammonia is liberated in the acidic hydrolysis of succinamopine lactam to succinopine lactam, this additional amide must be a primary amide. However, succinamopine cannot be a γ-glutaminyl derivative because the amide nitrogen would then be released as ammonia in the first transformation step to the succinamopine lactam instead of in the second step leading to succinopine lactam. Therefore, the primary amide of succinamopine and succinamopine lactam must be either at the α-carboxyl (isoglutamine structure) or in the nonglutamyl portion of the opine.

Spectroscopy. The NMR spectrum of succinopine lactam in D_2O at pD 2 (Figure 2a) contains a set of two coupled signals (d, 2.98 ppm; t, 4.88 ppm) in the ratio 2:1 and a second set of broad signals (2.48 and 4.55 ppm) in the ratio 4:1. The two low-field, single proton signals are in the range expected

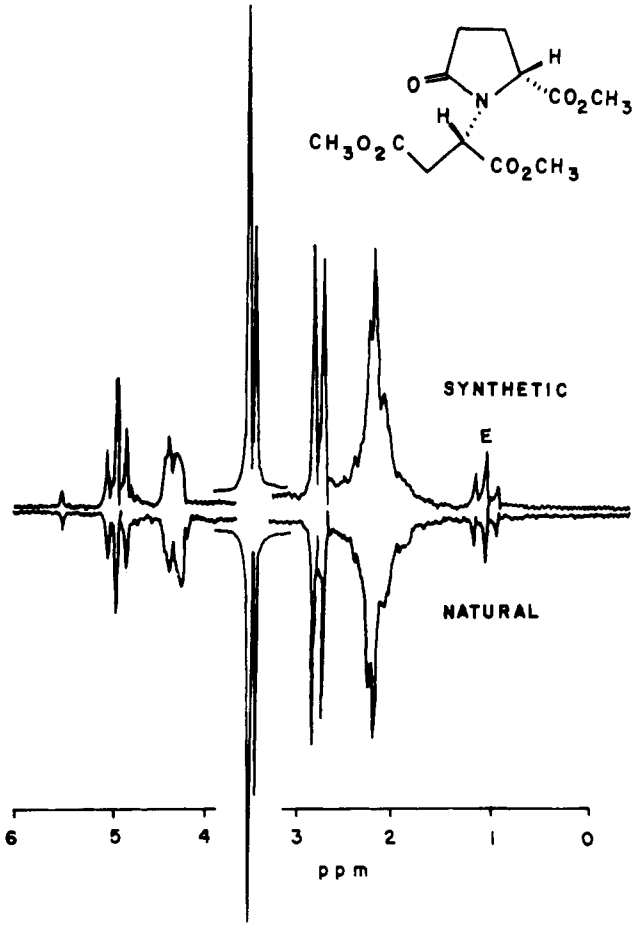


FIGURE 3: Proton NMR spectra (60 MHz) of synthetic and natural succinopine lactam trimethyl esters in $CHCl_3$ containing 1% ethanol. Ethanol signal marked E.

for α-proton signals of amino acids and *N*-acyl amino acids. These two signals must represent the two α-protons of the imino diacid group. Both α-H signals undergo an upfield shift on adjusting from pD 2 to 7 (Figure 2b). No further shift is observed at pD 13, indicating that only carboxyl groups are undergoing deprotonation, consistent with the nitrogen being present as a nonbasic lactam. The 4.88 ppm triplet undergoes the smaller shift (0.2 vs. 0.5 ppm), indicating that it is the signal of the α-H adjacent to the most acidic carboxyl (aspartyl α-carboxyl) that is already substantially ionized at pH 1.8 (Figure 1).

The areas, chemical shifts, and broadness of the signals at 2.48 and 4.55 ppm are consistent with the spectrum of glutamic acid lactam, in which the two γ-protons under the four-proton envelope are virtually coupled to the α-proton. The areas and splittings of the remaining signals at 2.98 and 4.88 ppm clearly indicate the presence of a three-proton ABX system with two nearly isochronous AB protons on a β-methylene group of the second amino acid moiety. This is consistent with the partial structure $YCH_2CH(N)CO_2H$ for the non-glutamyl moiety, in which Y is an anionically ionizing substituent containing only exchangeable protons. Although the two AB protons of the signal at 2.98 ppm are nearly isochronous, a slight asymmetry in the doublet is detectable at pD 2. The anisochronicity is enhanced in the 100-MHz spectrum of the triethyl ester in CCl_4 (Figure 2c), revealing the asymmetry of the system. By contrast, the trimethyl ester shows an apparent isochronous doublet in the NMR measured in $CHCl_3$ at 60 MHz (Figure 3). The NMR spectrum of succinopine lactam trimethyl ester has methoxyl resonances at 3.51 and 3.58 ppm (ratio 1:2), indicating the presence of three esterifiable carboxyls. Therefore, group Y must be a carboxylic acid, and succinopine lactam must be *N*-(1,2-dicarboxyethyl)-2-pyrrolidone-5-carboxylic acid (**2b** or diastereomer). The infrared spectrum of the ester in carbon tetrachloride shows a strong ester band at 1745 cm^{-1}, a tertiary γ-lactam (Pouchert, 1970) at 1705 cm^{-1}, and the absence of any NH or OH stretch above 3000 cm^{-1}.

The fast atom bombardment (FAB), positive-ion mass spectrum of succinamopine lactam (SAL) had the strongest peak at the 245 (SAL + H), with a weaker, ammoniated peak at 262 (SAL + NH_4) and a cluster ion at 489 (2 times SAL + H). Weaker cluster ions involving succinamopine lactam and the suspending glycerol were also observed. The FAB mass spectrum also contained a weak set of corresponding peaks due to the presence of succinopine lactam (SOL), including the mixed SAL + SOL + H cluster ion at 490. Electrophoretic analysis of the solution injected into the FAB mass spectrometer showed that some hydrolysis of succinamopine lactam to succinopine lactam plus ammonia had indeed taken place. Appropriate mass shifts were observed in the negative-ion FAB mass spectrum and in positive FAB on replacement of proton in the ion clusters by sodium after addition of NaCl to the glycerol suspension. Succinamopine, M_r 262, gave a positive FAB quasi molecular ion at 263 and a negative FAB ion at 261, and succinopine lactam, M_r 244, gave positive and negative FAB quasi molecular ions at 245 and 243. The high-resolution MS of succinopine lactam triethyl ester (molecular ion 329.1464) is in excellent agreement with elemental composition of $C_{15}H_{23}NO_7$ required for the triethyl ester of structure **2b**. Major electron-impact fragments were observed for loss of ethoxy (284), loss of carbethoxy (256), and extrusion of CO (182). The base peak at 83 is probably the pyrrolidonium fragment $C_4H_5O^+$. Succinamopine, succinamopine lactam, and succinopine lactam also gave molecular ions within 1 millimass unit of the expected value.

The FAB mass spectra of succinamopine and succinamopine lactam also contained a weak set of peaks, all shifted 14 mass units above those of succinamopine and succinamopine lactam, due to the presence of a homologue. The presence of a minor component (opine X), only slightly resolved electrophoretically from succinamopine isolated from TA519-8 crown gall tumor, was noted earlier, and the minor component was shown to be catabolized selectively as an opine by *A. tumefaciens* containing succinamopine-type plasmids (Chilton et al., 1984).

Synthetic Opines. Synthetic succinamopine was prepared by reductive condensation of α-ketoglutaric acid and (*S*)-asparagine. The product, after elution from a cation-exchange resin, showed the expected presence of diastereomers (u_{pic} 0.66 and 0.75), barely resolvable on electrophoresis at pH 2.8, the faster of which migrated with natural succinamopine. The mobility difference was enhanced on conversion to the lactam. Natural succinopine lactam comigrated with the more acidic, more mobile, synthetic diastereomer at pH 2.8. Each synthetic diastereomeric pair (succinamopine, succinamopine lactam, and succinopine lactam) has a greater difference between first dissociation constants than between second dissociation constants; consequently, succinamopine ($pK_{a1} < 1.7$) and isosuccinamopine are only slightly resolved at pH 2.8, while the two pairs of diastereomeric lactams (pK_{a1} = ca. 2.3) are well resolved at this pH but not resolved above pH 4. This difference in pK_{a1} was used to fractionate synthetic succinopine lactam and isosuccinopine lactam on an anion-exchanger by suppression of ionization with a formic acid gradient. As expected, the weaker acid isosuccinopine lactam emerged first

Table II: Circular Dichroism of N-Substituted Pyroglutamic Acids

compound	molar ellipticity pH 1	pH 5	pH 10	methyl ester[a]
(*S*)-glutamic acid lactam (**5a**)	14.7	20.6	20.6	17.2[b]
($S^{Asp}R^{Glu}$)-succinopine lactam (**2b**)	−6.0	−29.3	−46.3	−23.2
($S^{Asp}S^{Glu}$)-isosuccinopine lactam	5.8	22.3	23.8	>2
natural succinopine lactam (**2b**)				−24.1
nopaline lactam (**4**)	−28.4		−97.0	
agropinic acid (**5b**)	17.8	30.3	30.2	

[a] Methanol solvent. [b] Goodman et al. (1969).

at ca. 3.5 M formic acid and succinopine lactam at ca 4.8 M. Both of the resolved synthetic lactams and their trimethyl esters gave appropriate FAB-MS quasi molecular ions and high-resolution molecular ion elemental composition.

One of the two resolved lactams had electrophoretic mobility, circular dichroism (Table II), and pH-dependent NMR between pH 1 and 13, identical with succinopine lactam derived from tobacco tumor. The circular dichroism (Table II) and proton NMR (Figure 3) of the trimethyl ester of this synthetic diastereomer were also identical with those of the trimethyl ester of succinopine lactam derived from tobacco tumor, confirming the structure of succinopine lactam as *N*-(1,2-dicarboxyethyl)-2-pyrrolidone-5-carboxylic acid. Isosuccinopine lactam trimethyl ester (NMR methoxyl signals at 3.60 and 3.63 ppm, area ratio 2:1) is readily distinguishable from natural succinopine lactam trimethyl ester (methoxys at 3.54 and 3.60 ppm, area ratio 1:2) by the reveral of the 2:1 methoxy signals in the NMR, as well as by the opposite sign of the circular dichroism extremum at 215 nm (Table II).

The same synthetic lactams were also prepared by reductive condensation of α-ketoglutaric acid and (*S*)-aspartic acid via succinopine (**1b** and diastereomer). An amorphous mixture of **1b** and diastereomer was prepared earlier by an alkylation method (Karrer & Brandenberger, 1951), but no chromatographic data were reported. Succinopine diastereomers are only partially resolved on very long electrophoretic runs at pH 2.8 (u_{pic} 0.75 and 0.76) and are not resolved from succinamopine. For these reasons, no attempt was made to determine whether succinopine is present in tumors or whether it is fermented by *A. tumefaciens*.

Selective Catabolism of Diastereomers of Synthetic Opines and Their Lactams. The mixture of diastereomeric succinamopines prepared from (*S*)-asparagine was incubated in liquid culture with *A. tumefaciens* strains A518, A519, and A532, previously shown to have succinamopine-type Ti plasmids (Chilton et al., 1984). A volume of solution originally containing 50 μg of opine was analyzed electrophoretically with detection by the silver nitrate–mannitol reagent capable of detecting 5 μg or less. All these strains totally consumed the electrophoretically more mobile succinamopine diastereomer (u_{pic} 0.75, pH 2.8) within 72 h. The other diastereomer (isosuccinamopine) was undiminished after 150 h of incubation. Control strains A136 (lacking Ti plasmid), A208 (nopaline-type Ti plasmid), and A277 (octopine-type Ti plasmid) did not detectably diminish either succinamopine or isosuccinamopine within 150 h. The selectively catabolized diastereomer had the same electrophoretic mobility as natural succinamopine.

Similarly, strains A518, A519, and A532 selectively consumed the more mobile succinamopine lactam diastereomer (u_{pic} 0.98, pH 2.8) that comigrates with natural succinamopine lactam and did not consume the less mobile diastereomer (isosuccinopine lactam) within 150 h. Strains A136, A208, A277, and A281 (agropine/leucinopine-type Ti plasmid) did not diminish either succinamopine lactam diaastereomer within 150 h. The more mobile diastereomer of succinopine lactam (u_{pic} 0.98, pH 2.8) was also selectively consumed by strains A518, A519, and A532 within 72 h, while the less mobile diastereomer remained undiminished after 150 h. Strains A136, A208, A277, and A281 did not diminish either diastereomer within 150 h. The three succinamopine-type strains consumed natural succinamopine, succinamopine lactam, and succinopine lactam within 72 h, while the four control strains did not consume the natural opines within 150 h.

The synthetic diastereomeric mixture of succinamopines was also capable of serving as the sole carbon source for growth of three *A. tumefaciens* strains (A518, A519, and A532) previously shown to have succinamopine-type Ti plasmids (Chilton et al., 1984). The isogenic control strain A136, lacking the Ti plasmic, did not grow on either natural or synthetic succinamopine. The synthetic lactams also served as the sole carbon source for the same succinamopine-type strains, but not for control strain A136.

Absolute Stereochemistry of the New Opines. Succinopine lactam was prepared by methods involving one center of known chirality and creation of one new center, leading to diastereomeric pairs in each case, but a different set of the four diastereomers paired in each case (Table I). In some cases, the diastereomers were resolved before subjection to liquid fermentation analysis; in other cases, fermentability of each diastereomer in a pair was assessed by pH 2.8 electrophoresis after liquid fermentation. For convenience, the two chiral centers of succinopine lactams are referred to as the aspartyl and the glutamyl chiral centers. The method of synthesis and selectivity of fermentation clearly indicate that natural succinopine lactam has (*R*)-glutamyl and (*S*)-aspartyl chirality; hence, natural succinamopine and succinamopine lactam have this chirality also.

Circular Dichroism of Opine Lactams. Glutamic acid lactam (**5a**) and its N-substituted derivatives have a strongly chiral amide n–π^* transition at 215 nm (Goodman, 1969). The ellipticity is dependent on the extent of ionization of the carboxyl group on the chiral center, the absolute value of the ellipticity being greater for the anion than for unionized carboxylic acid. For the N-substituted lactams examined (Table II), the glutamyl center dominated over chiral centers in the other N-substituent in determining the sign of the ellipticity and the direction of ellipticity shift between pH 1 and 10. A positive ellipticity and a positive shift on changing pH from 1 to 10 is found for N-substituted (5*S*)-2-pyrrolidone-5-carboxylic acids and negative ellipticity for N-substituted (5*R*)-2-pyrrolidone-5-carboxylic acids. The *R* chirality assigned to the glutamyl center of succinopine lactam on the basis of comparative fermentation of diastereomers is consistent with the observed negative ellipticity of natural succinopine lactam.

Discussion

We have shown by chemical evidence, spectroscopy, and fermentation that succinamopine (**1a**) is the prototype of a new group of opines present in tobacco tumor incited by *A. tumefaciens* strains A518, A519, AT181, and EU6. The derived lactams meet the catabolic definition of an opine, but we have not yet demonstrated their presence in tumors. Lactamization is so facile that succinamopine lactam may be encountered in tumor extracts as a nonenzymatic transformation product. It is thus not remarkable that bacterial strains containing succinamopine-type plasmids are able to catabolize succinamopine lactam as well. A similar situation exists in *A. tu-*

mefaciens strains containing mannopine-type plasmids, which catabolize agropinic acid, the analogous lactam derived from mannopine. They possess at least an agropinic acid permeasease, and probably a catabolase, different from mannopine permease and catabolase (Chilton & Chilton, 1984). Succinamopine-type bacterial strains also catabolize succinopine lactam. No evidence exists for the presence of succinopine lactam in tumors; its catabolism may be via the catabolase for the structurally closely related succinamopine lactam.

Evidence has been presented (Chang & Chen, 1983) for the existence of a mixed imino diacid chromatographic fraction from pinto bean tumors incited by *A. tumefaciens* strains 181 and EU6, which on oxidation with $KMnO_4$ liberates glutamic acid, glutamine, aspartic acid, asparagine, serine, glycine, and alanine, among other amino acids. The evidence suggests the probable presence of several further opines in which glutamic acid is a partner. Although no specific imino diacid was isolated from the complex, it was observed that a crude enzyme extract from pinto bean tumor incited by strain 181 could convert radioactive (*S*)-asparagine in the presence of α-ketoglutaric acid and NADPH into a substance eluting chromatographically in the same volume as the imino diacid fraction from tumors. This substance, asparoginopine, may be identical with succinamopine.

All of the nitrogenous opines for which absolute stereochemistry is known have in common a recognizable amino acid moiety of *S* chirality; this includes the mannityl opines. In the imino diacid subclass of opines (lysopine, octopine, histopine, nopaline, and succinamopine), where a second amino acid moiety sharing the same nitrogen atom is recognizable, the second amino acid has *R* chirality, resulting in erythro relative stereochemistry of the two chiral centers. Because of the configurational identity around the imino diacid group, some cross catabolism of octopine by nopaline-type plasmids (Petit & Tempé, 1978) and of nopaline by succinamopine-type plasmids (Sciaky et al., 1978) has been observed. There appears to be some tolerance of opine structural variation in the plasmid-borne imino diacid catabolase systems, as has been observed more extensively for mannityl opine type plasmids (Chilton & Chilton, 1984), but strict requirement for *SR* chirality of the imino diacid has been observed so far for opine catabolism by octopine, nopaline, and succinamopine-type plasmids.

The biosynthetic significance of erythro (*SR*) stereochemistry observed in all of the imino diacid opines may reflect a common pattern of origin via enzymatic reduction of the Schiff base of a readily available amino acid and a common carbonyl metabolite. It is probably the amino acid moiety having *S* chirality in these opines that is derived directly from the plant's pool of natural (*S*)-amino acids, while the amino acid moiety having the uncommon *R* chirality may be created in the reduction step from the common α-keto acids. All of the imino diacids found to date may be viewed as reductive conjugates of the two abundant α-keto acids pyruvic acid and α-ketoglutaric acid.

Acknowledgments

We thank Lucy Kayes for maintaining tissue cultures of tobacco tumors and Haris Chrysokopoulis for preparing nopaline lactam.

Registry No. **1a**, 88194-24-5; **1b**, 90245-73-1; **2a**, 89444-68-8; **2b**, 89444-69-9; **3**, 22350-70-5; **4**, 86630-17-3; **5a**, 98-79-3; **5b**, 74474-75-2; (*SS*)-isosuccinopine lactam trimethyl ester, 90245-74-2; (*SR*)-succinopine lactam trimethyl ester, 90245-75-3; (*SS*)-succinopine lactam, 90245-76-4; (*RS*)-succinopine lactam, 90245-77-5; (*RR*)-succinopine lactam, 90245-78-6.

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