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Saccharopine, a New Amino Acid in Baker's and Brewer's Yeast

II. Structure and Synthesis

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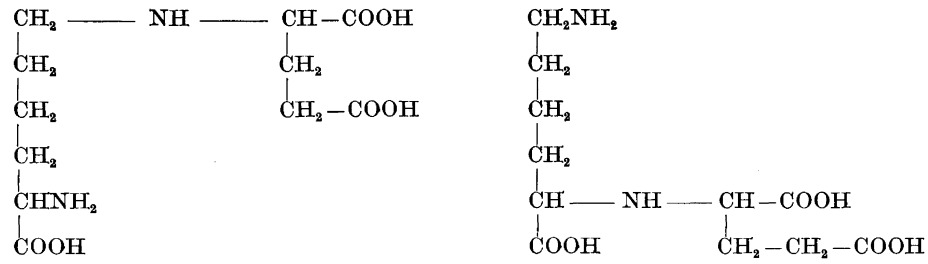
The combined chemical and biochemical data, presented in other communications of this series, suggest (I) or, less likely, (II), as plausible structures for the new yeast amino acid *saccharopine*. The former compound is synthesized as a mixture of diastereoisomerides by Strecker-reaction of N^2-(*p*-toluenesulphonyl)-L-lysine, potassium cyanide and 2-oxoglutaric acid. The route involves condensation of the three components, acid hydrolysis, decarboxylation and removal of the protecting group. Fractional crystallization affords synthetic material identical with natural saccharopine, and, in addition, its diastereoisomeride.

The synthetic route, together with rotation data for the two isomerides, unequivocally establishes (I), with L-centres in both the lysine and glutamic acid portions, as the structure of the new, natural amino acid, hence denoted L-saccharopine. The synthetic diastereoisomeride, possessing D-configuration at the glutamic acid centre, is accordingly named D-*allo*saccharopine. A synthetic by-product is tentatively formulated as a derivative of pyroglutamic acid.

The natural occurrence of related imino acids and the biogenesis of saccharopine are briefly discussed.

The combined chemical and biochemical evidence, presented in preceding communications [1,2], can be rationalized in terms of formula (I) or (II) as plausible expressions for the new amino acid, saccharopine, isolated from baker's yeast. The assay for α-monoamino monocarboxylic acids, developed in this laboratory [3], gave results favouring structure (I) rather than (II). Hence, efforts were directed towards synthesis of the former.

The rather few heretofore known N-carboxyalkylamino acids are almost all of the α,α′-type and have largely been synthesized by four different methods. (i) Reductive alkylation of amino acids with keto acids has been employed in, *e.g.*, the preparation of *allo*octopine from L-arginine and pyruvic



I II

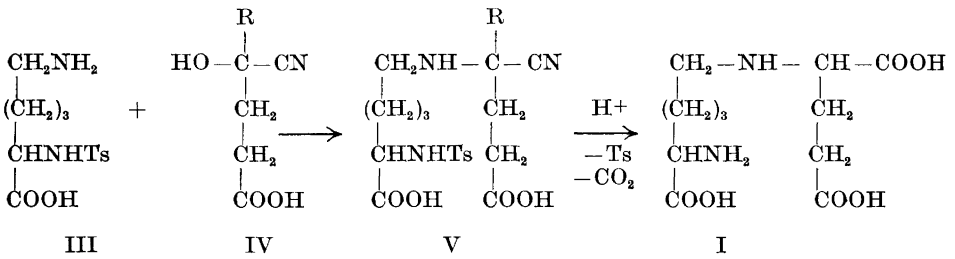
acid [4], while attempts to produce octopine in a similar fashion from 2-oxo-5-guanidovaleric acid and alanine met with no success [5]; other failures to achieve similar reactions have been reported [6]. (ii) N-alkylation of amino acids with halo-acids has been repeatedly utilized in the syntheses of imino acids, *e.g.* optically active octopine [4,5] and N-(1-carboxyethyl)-substituted alanine [7], aspartic and glutamic acid [8]; (iii) Strecker-synthesis, involving the reaction between a carbonyl compound, cyanide and an amino acid, has yielded such amino acids as N-carboxymethyl-L-glutamic acid [9] and a mixture of diastereoisomeric N-(1-carboxyethyl)-glutamic acids [10]. (iv) Addition of an amino acid or ammonia to an unsaturated acid constitutes a known route to certain symmetric imino acids [11–13].

Common for the first and the two last methods is the introduction during synthesis of at least one asymmetric centre resulting in the formation of diastereoisomer mixtures of unpredictable compositions, whereas in the second method precise kinetic data are required for unequivocal prediction of the stereochemical course of the reaction.

In the synthetic approach to structure (I) various modifications of the first three of the above general methods were attempted. First, mixtures of 2-oxoglutaric acid and the copper complex of L-lysine or N^2-(*p*-toluenesulphonyl)-L-lysine [14,15] were subjected to hydrogenation over noble metal catalysts; in both cases, however, only reduction of the keto-acid to 2-hydroxyglutaric acid was observed. Subsequently, the synthesis of DL-5-(3-formylpropyl)-hydantoin, with a view to its application in reductive alkylation of L-glutamic acid, was unsuccessfully attempted. No better results attended experiments on the alkylation of ethyl L-glutamate with DL-5-(4-bromobutyl)-hydantoin [16]. However attractive for stereochemical reasons, further attempts to utilize optically active glutamic acid or esters hence were abandoned. As an obvious alternative, alkylation of N^2-(*p*-toluenesulphonyl)-L-lysine methyl ester [14,32] with ethyl DL-2-bromoglutarate [33], followed by saponification and detosylation, was attempted. A minute amount of a product, indistinguishable from saccharopine on paper chromatograms, was present in the reaction mixture, yet accompanied by considerable quantities of lysine and two nitrogen-free acids, probably 2-ethoxyglutaric acid and 1,2-cyclopropanedicarboxylic acid, both formed from the halo acid by metathetic and intramolecular cyclization reactions, respectively. The unsatisfactory course of this substitution reaction, together

with some uncertainty as to the structure of the resulting product *, made it natural at this stage to explore the merits of the Strecker-synthesis for the preparation of (I).

According to the following scheme, interaction between N^2-(*p*-toluenesulphonyl)-L-lysine (III, Ts = p-$CH_3C_6H_4SO_2$) and the cyanohydrin of succinaldehydic acid (IV, R = H) should provide a promising route to a mixture of diastereoisomerides of (I) (*cf.* foot-note, p. 754). The latter aldehyde has



in fact been used previously in similar reactions with simple amines [18]. After a few preliminary experiments, however, it was found advantageous to replace the easily polymerizing succinaldehydic acid with the readily accessible 2-oxoglutaric acid in the above reaction scheme. In a model experiment, where the latter keto-acid was treated with ammonia and potassium cyanide, the formation of glutamic acid was paperchromatographically established. Subjected to similar conditions, (III) afforded a 65 % yield of the cyanohydrin (V, R = COOH) which, because of its rather labile character, was transformed without further purification into a semicrystalline product on acid hydrolysis.

After detosylation with hydrogen bromide in glacial acetic acid three ninhydrin-positive spots on the paper chromatograms could be tentatively assigned to lysine, saccharopine and its transformation product, discussed in a preceding communication [1]. Diastereoisomerides of the two last amino acids were later found to be inseparable upon paper chromatography. Probably, the notable quantity of lysine present in the reaction mixture originated from N^2-(*p*-toluenesulphonyl)-L-lysine, formed by concomitant acid fission of (V, R = COOH) during hydrolysis and decarboxylation of the latter. Attempts to suppress the quantities of lysine and the transformation product of saccharopine by employing alkaline hydrogen peroxide as a hydrolyzing agent and sodium in liquid ammonia for removal of the protecting group were unsuccessful.

On a preparative scale, the total amino acid fraction was isolated by means of a strongly acid ion exchange resin and subsequently freed of lysine by passage through a weakly basic resin in the acetate form. The acid eluate from the latter contained only the two amino acids, tentatively regarded as

* Cocker [17] actually utilized N-alkylation of N-benzenesulphonyl derivatives of α-monoamino acids as an intermediate stage in the preparation of sarcosine and simple homologues.

saccharopine and its transformation product. Repeated precipitations with ethanol from aqueous solutions of the mixture at pH 3.5 yielded a paperchromatographically homogeneous, crystalline product, possessing the same R_F-value and elemental composition as saccharopine isolated from yeast. However, considerable deviations in the infra-red spectra and optical rotations of this synthetic material clearly indicated its character as a mixture of two diastereoisomerides. Fractional crystallization of the latter was facilitated by seeding with saccharopine, and after a few recrystallizations from water a synthetic sample was obtained with correct elemental composition and pos-

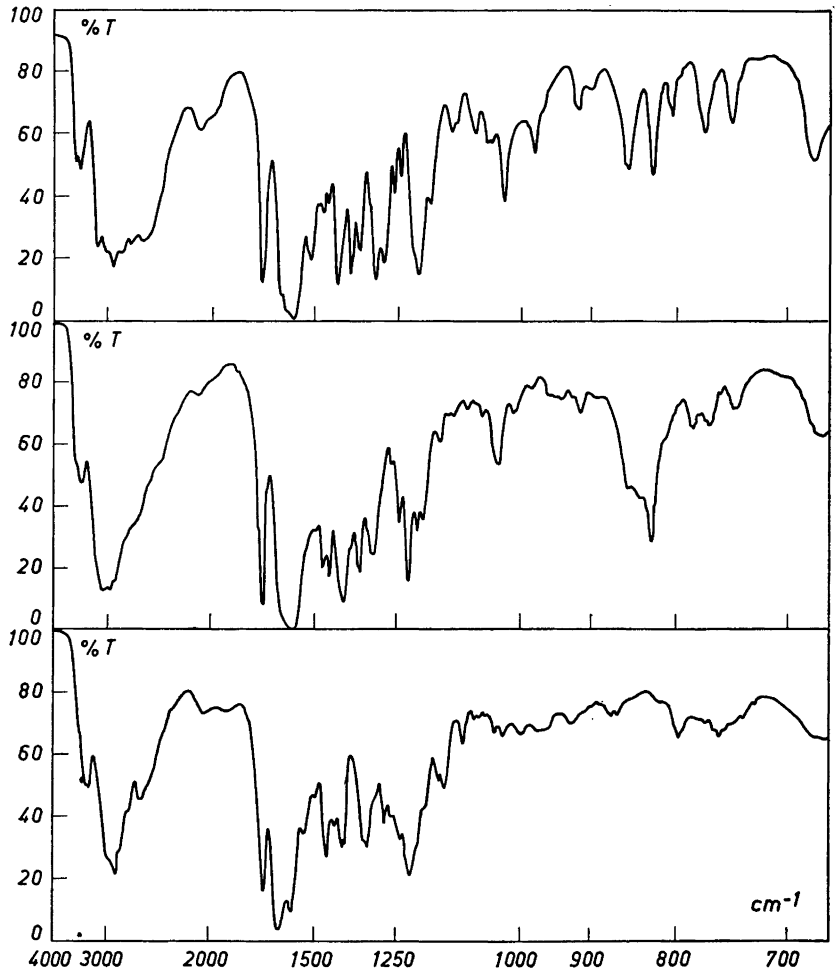
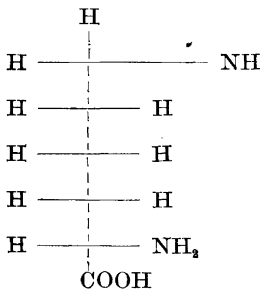


Fig. 1. Infra-red spectra (in KBr pellets). Upper curve: synthetic L-saccharopine (identical with that of the natural amino acid). Middle curve: synthetic D-*allo*saccharopine. Lower curve: cyclic by-product (VII), probably mixture of two diastereoisomerides.

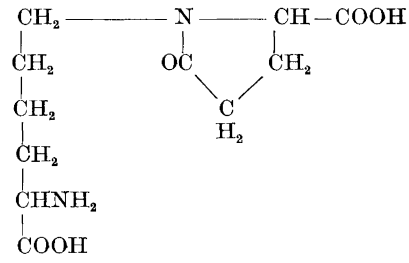
sessing the same rotation, within experimental errors, as the natural amino acid. Furthermore, R_F-values, infra-red spectra (Fig. 1) and X-ray diffraction patterns of the two preparations were identical and no melting point depression was observed. Hence, apart from the absolute configuration around the second asymmetric centre, the above synthesis establishes (I) as the correct structure for the new amino acid saccharopine.*

Repeated recrystallizations of the mother liquor material afforded the more soluble diastereoisomeride with correct composition and a markedly different infra-red spectrum. Judged from the change in specific rotation on successive recrystallizations, this isomeride also was obtained in optically pure state.

Isolation of both synthetic diastereoisomerides rendered determination of their absolute configuration possible on basis of rotation data. By application of the principle of optical superposition to the two isomerides, the contribution of each asymmetric centre to the rotation was calculated. The values are presented in Table 1 together with rotation data for L-lysine and some appropriate derivatives of L-glutamic acid. From these considerations it can be safely concluded that saccharopine possesses L-configuration at both asymmetric centres as depicted in (VI), whereas the more soluble synthetic diastereoisomeride contains a D-centre in the glutamic acid portion of the molecule.



VI



VII

According to the proposal of Vickery [34], the configurational designation in compounds given trivial names and containing two α-amino carboxylic acid groupings attached to dissimilar carbon chains shall be that of the α-carbon atom of the smaller of the two chains. Adopting this rule, the natural amino acid is to be denoted L-saccharopine, and the diastereoisomeride, differing in the configuration of the glutamic acid portion, is D-*allo*saccharopine.

* The possibility of the sterically unfavourable reaction between (III) and (IV) taking place at the ionized sulphonamido grouping, present in the strongly alkaline reaction mixture, in analogy with the reported reaction between sulphonamides and aliphatic aldehyde cyanohydrins [35], has been considered. In order to exclude the latter reaction in the present case, N^6-(*p*-toluenesulphonyl)-L-lysine was subjected to reaction with (IV) under conditions similar to those employed in the synthesis of saccharopine. No reaction occurred as evident from the presence of lysine as the sole amino acid, subsequent to hydrolysis and detosylation. Again, a Strecker-synthesis between (IV) and L-lysine furnished a moderate yield of the diastereoisomeric saccharopines.

Table 1. Optical rotation data for saccharopine and its diastereoisomeride, and contributions of the individual asymmetric centres to the rotation.

Compound	Measured $[M]_D$ in 0.5 N HCl	$[M]_D$ Lysine portion	$[M]_D$ Glutamic acid portion
L-Saccharopine	+ 93°	+ 45° *	+ 48° **
D-*allo*Saccharopine	−2°	+ 45°	− 48°

* For comparison: $[M]_D$ of L-lysine in 0.5 N HCl: + 38°; ** $[M]_D$ of L-glutamic acid: + 47° (0.5 N HCl), and for the following N-substituted L-glutamic acids: methyl + 40° (hydrochloride in H_2O) [19], ethyl + 38° [20], *n*-propyl + 41° [20], *n*-butyl + 39° [20] (the abstract of Ref.[20] does not state the character of the solvent).

During the present studies it was unsuccessfully attempted to prepare the isomeric compound (II) by Strecker-syntheses involving 2-oxoglutaric acid and N^6-benzyloxycarbonyl-L-lysine or N^6-(*p*-toluenesulphonyl)-L-lysine [21] (*cf*, footnote, p. 754). Attempts to effect reductive alkylation of the latter derivative with 2-oxoglutaric acid met with no better success.

From the combined mother liquors of the diastereoisomeric saccharopines the more soluble transformation product, invariably formed during the syntheses of saccharopine, was isolated in crystalline and paperchromatographically homogeneous form. Rotation data, however, suggested that the isolate was a difficultly separable mixture of diastereoisomerides. Earlier evidence [1], now supplemented with the elemental composition $C_{11}H_{18}O_5N_2$, ultra-violet absorption data, and a positive response to the assay for α-monoamino monocarboxylic acids [3], is consistent with a lactam structure such as (VII) for the by-product, comparable to that present in pyroglutamic acid. In analogy, heat treatment of N-(D-1-carboxypropyl)-L-glutamic acid has been shown elsewhere to cause conversion of the latter imino acid into an anhydride, formulated as the corresponding pyroglutamic acid derivative [8].

Upon heating in the dry state to temperatures close to the melting points, the diastereisomeric saccharopines were converted into cyclized products, indistinguishable from the lactams described above. Both isomerides were completely and irreversibly transformed into cyclic derivatives on heating with water at 125°, a behaviour deviating notably from the reversibility observed in strong acid or alkali (*cf.* Ref.[1]).

The structure of L-saccharopine does not preclude its biosynthesis from L-lysine and 2-oxoglutaric acid, but is, on the other hand, equally consistent with its biological formation from L-glutamic acid and L-2-amino-5-formylvaleric acid (α-aminoadipic-δ-semialdehyde). It is interesting in this connexion that the latter aldehyde most likely is an actual intermediate in the biosynthesis of lysine from α-aminoadipic acid in *Neurospora* and yeast [22,23].

Imino acids have occasionally been suggested as possible intermediates in transamination reactions [24] though the available experimental evidence is meagre. Apart from cyclic types and simple N-alkylated amino acids only a very limited number of imino acids have thus far been encountered in Nature. Octopine was isolated from *Octopus* muscle in 1927 [25] and its constitution as N^2-(D-1-carboxyethyl)-L-arginine established a decade later [26,27] (*cf.* also Ref.[5]).

Recently, a French group [28] established the structure of lysopine, an amino acid occurring in crown galls from several higher plants, as N^2-(D-1-carboxyethyl)-L-lysine.

EXPERIMENTAL

Melting points are uncorrected and determined in capillary tubes in an Anschütz-Hershberg apparatus with a rate of heating of about 2° per min. Rotations are measured in a 1 dm tube. Infra-red spectra are determined in potassium bromide pellets on a Perkin-Elmer "Infracord"-instrument.

N^6-Benzyloxycarbonyl-L-lysine. Although in principle the same as the published method (*cf. e.g.* Ref.[29]), the following synthetic procedure is believed to possess certain advantages. L-Lysine monohydrochloride (9 g) was boiled for 30 min with cupric carbonate (3.5 g) and water (100 ml). Magnesium oxide (6 g) was added to the filtrate, and a solution (13 ml) of benzyloxycarbonyl chloride (56 mequiv.) in toluene was added in small portions in the course of 30 min. to the ice-cooled and stirred suspension. After stirring for another 2 h at room temperature, the mixture was cooled in ice, filtered, and the precipitate was washed with water, ethanol and ether. A stirred suspension of the solid in 1 N HCl (325 ml) was saturated with hydrogen sulphide, whereupon the mixture was heated to boiling, filtered and adjusted to pH 5 with conc. NH_3. The resulting solid was recrystallized from water (1 700 ml) and dried at 80°. There resulted 11.2 g (80 %) of the pure compound, m.p. 240—244° (decomp.), $[\alpha]_D^{23}$ + 13.5° (*c* 1.4, 0.1 N HCl). Literature value: $[\alpha]_D$ + 14.4° (*c* 1.6, 2 equiv. HCl in H_2O) [29].

N^2-(p-Toluenesulphonyl)-N^6-benzyloxycarbonyl-L-lysine. This derivative was prepared as previously described [30,31] with the minor modifications that ether was used as a solvent for the sulphonyl chloride and chloroform for extraction of the reaction product. The latter was recrystallized from benzene and dried at 80°. A yield of 89 % of unsolvated (*cf.* Ref.[15]), pure product was obtained, m.p. 121—122°, $[\alpha]_D^{23}$ + 13.5° (*c* 2.2, CH_3OH). Lit. values: m.p. 123—124° [30], 126° [15]; no rotation reported.

N^2-(p-Toluenesulphonyl)-L-lysine. The preparation of this compound by hydrogenolysis of the foregoing derivative has previously been reported [14], yet without experimental details. In the present case, N^2-(*p*-toluenesulphonyl)-N^6-benzyloxycarbonyl-L-lysine (12 g) was dissolved in a mixture of acetone (125 ml) and conc. HCl (4.5 ml), and treated, in the presence of palladium black (0.85 g), at 50° with a stream of hydrogen until the evolution of CO_2 ceased. Then, 4 N HCl (10 ml) and water (60 ml) were added, the catalyst was filtered off and acetone removed from the filtrate, which was finally extracted twice with ether. After being adjusted to pH 6 with conc. NH_3, the aqueous phase deposited the somewhat impure reaction product (7.6 g or 91 %; Ref.[14] lists 90 %). A pure specimen was obtained in beautifully crystalline form from water, m.p. 262—265° (decomp.), $[\alpha]_D^{22}$ −50.9° (*c* 2.0, 0.5 N NaOH). Lit. values: 263—264° (decomp.)[14], $[\alpha]_D^{19}$ −51.6° (*c* 2.3, 0.5 N NaOH) [31].

N^2-(p-Toluenesulphonyl)-L-lysine methyl ester hydrochloride. As elsewhere described [14], treatment of the above amino acid derivative with anhydrous methanol and HCl afforded the methyl ester hydrochloride in a yield of 78 %. A correctly analyzing specimen was obtained from a mixture of methyl acetate and pentane, m.p. 143—144°, $[\alpha]_D^{22}$ + 9.5° (*c* 1.0, CH_3OH).* Lit. values: m.p. 146—147° [14], 148—150° [32]; $[\alpha]_D^{22}$ −10.2° (*c* 4.0, H_2O)[32]**.

Diethyl DL-2-bromoglutarate. This ester, used in preliminary attempts to synthesize saccharopine, was prepared in 94 % yield according to the directions given by Schwenk and Papa [33]. Redistillation of a center cut from the first distillation afforded a pure sample, b.p. 89—92° at 0.3 mm, n_D^{20} 1.4591. (Found: C 40.25; H 5.88; Br 29.76. Calc. for $C_9H_{15}O_4Br$: C 40.46; H 5.66; Br 29.92).

* This product proved identical with a specimen described [14] and kindly furnished by Dr. D. T. Elmore.

** The notable change in rotation with solvent has been verified in this laboratory.

Synthesis of a mixture of L-*saccharopine and* D-allo*saccharopine.* In order to determine the optimal conditions for the following synthesis, the dissociation constant for the cyanohydrin of 2-oxoglutaric acid was experimentally determined by electrometric titration of a mixture of the acid and potassium cyanide. The found value of ca. 10^{-3}, together with the acid dissociation constant of hydrogen cyanide, enabled calculations of the best conditions for the synthesis. To 2-oxoglutaric acid (730 mg), dissolved in water (15 ml) and 1.2 N NaOH (8.9 ml), potassium cyanide (2.5 g) was added and pH was adjusted to 11.3 with 4 N HCl. This solution was mixed with another, prepared by dissolving N^2-(*p*-toluenesulphonyl)-L-lysine (1.75 g) in water (15 ml), adding 1.2 N NaOH (5.25 ml) and adjusting the pH to 11.4 with 4 N HCl. The mixture was kept in a closed vessel for 18 h at 50° and after cooling acidified to pH 1.9 with conc. HCl, when the amino-cyanohydrin (V) separated in 65 % yield (1.5 g) as a colourless, crystalline product.

Without further purification, the latter (1.5 g) was dissolved in conc. HCl (25 ml) and set aside at room temperature for 16 h. Then water (25 ml) was added and the solution was refluxed for 2 h. After evaporation to dryness *in vacuo* and careful removal of the last traces of acid, the dry, semisolid mass was dissolved in liquid ammonia (100 ml), and sufficient metallic sodium (*ca.* 0.5 g) added to produce a persistent blue colour. Thirty min. later enough ammonium acetate was added to cause a change to yellow colour and the ammonia was allowed to evaporate. The residue was dissolved in water (10 ml), treated with charcoal, filtered, and pH in the filtrate adjusted to 3.8 with 4 N HCl. After two extractions with ether and dilution to a volume of 100 ml, the aqueous phase was applied to a column (36 cm × 1.5 cm) of a strongly acid ion exchange resin (Zerolit 225) in the hydrogen form. The column was washed with water and the entire amino acid fraction eluted with 1 N NH_3. The dry eluate residue (650 mg), dissolved in CO_2-free water (50 ml), was then applied to a weakly basic resin (Amberlite IR-4B, 24 cm × 1.5 cm) in acetate form, and after washing with water, 2 N HCl was used for elution. The eluate was concentrated to dryness *in vacuo* to give a semi-crystalline residue (470 mg), which on paper chromatography gave one spot attributable to the isomeric saccharopines, and another spot representing their transformation products.

A fraction (272 mg) of the above product was dissolved in water (2 ml) and treated with charcoal. After filtration and concentration to 0.8 ml, the addition of ethanol (0.5 ml) caused the precipitation of a finely crystalline product (65 mg). Four similar precipitations yielded a preparation (18 mg) of the diastereoisomer mixture which on paper chromatography was homogeneous and indistinguishable from authentic saccharopine, $[\alpha]_D^{25}$ + 14° (*c* 2.0, 0.5 N HCl, 0.5 dm tube) (Found (corrected for 0.77 % of ash): C 47.95; H 7.47; N 9.93. Calc. for $C_{11}H_{20}O_6N_2$: C 47.82; H 7.30; N 10.14).

Separation of diastereosiomerides. A solution in water (7.5 ml) of the above-described semi-crystalline residue from the HCl-eluate (1.25 g) was treated with charcoal, filtered, adjusted to pH 3.7 with 4 N ammonia and concentrated to a volume of 6 ml. Addition of ethanol (9 ml) caused the precipitation of a crystalline solid (340 mg), which according to paper chromatography was virtually free of the conversion products of the saccharopines. The solution of this product (230 mg) in water (6 ml), was treated with charcoal to give a colourless solution, which was concentrated to half of the volume, seeded with natural L-saccharopine, and kept at 4° for 6 days. The crystalline compound (77 mg, $[\alpha]_D^{23}$ + 25.7° (*c* 1.3, 0.5 N HCl)) was subsequently subjected to three similar recrystallizations to give a pure specimen (27 mg), m.p. 240–248° (decomp.), alone or in admixture with natural saccharopine, (Found: C 47.76; H 7.24; N 10.18) with $[\alpha]_D^{23}$ + 32.2° (*c* 1.0, 0.5 N HCl), compared to the value $[\alpha]_D^{23}$ + 33.6° (*c* 1.0, 0.5 N HCl) determined for pure, authentic L-saccharopine [1]. Furthermore, coinciding IR-spectra and X-ray diffraction patterns served to establish the identity of the two compounds.

On concentration to a small volume, the mother liquor from the first crystallization deposited a colourless solid (62 mg) with $[\alpha]_D^{24}$ + 4.2° (*c* 0.9; 0.5 N HCl). Once recrystallized from water the product had a specific rotation of −0.6°, and renewed recrystallizations afforded an end-product (17 mg), m.p. 235–250° (decomp., after preliminary sintering at 215–217°) with $[\alpha]_D^{23}$ −0.8° (*c* 0.4, 0.5 N HCl) (Found: C 47.51; H 7.46; N 10.28), and believed to represent pure or nearly pure D-*allo*saccharopine.

Isolation of a cyclic by-product. The combined mother liquors from the above described isolations of L- and D-*allo*-saccharopine were passed through a strongly acid ion exchange resin and eluted with ammonia to give a brownish glassy mass (600 mg). This was dissolved in water and decolourized by filtration through a small column of alumina. Addition of ethanol to the filtrate resulted in separation of the ammonium salt of the unknown by-product (180 mg). The filtrate was adjusted to pH 2.5 and acetone was added to induce crystallization of the cyclic by-product (230 mg), $[\alpha]_D^{21}$ + 7.0° (*c* 0.9, H_2O). Two additional recrystallizations from water and acetone gave material, m.p. 245–248° (decomp), with unchanged rotation ($[\alpha]_D^{23}$+6.8° (*c* 1.0, H_2O); $[\alpha]_D^{23}$+18.2° (*c* 0.9, 1 N HCl)) (Found: C 50.59; H 7.10; N 10.96. Calc. for $C_{11}H_{18}O_5N_2$: C 51.15; H 7.03; N 10.85). The infrared absorption curve is depicted in Fig. 1. The ultra-violet absorption spectrum had a molecular extinction of 4 200 at 205 mμ, compared to 200 for L-saccharopine, supporting the cyclic structure (VII).

The above rotation value, corresponding to molecular rotations of + 17.4° and + 46.5° in water and acid, on comparison with the $[M]_D$-values + 19.4° (H_2O) and + 37.9° (HCl) for L-lysine and −15.4° (H_2O) for L-pyroglutamic acid, suggest that the analyzed specimen may be a difficultly separable mixture of about equal parts of two diastereoisomerides.

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