98. Studies in the Biochemistry of Micro-organisms

71. Fumaryl-*dl*-alanine (Fumaromono-*dl*-alanide), a Metabolic Product of *Penicillium resticulosum* sp.nov.

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A systematic investigation of the production of antibacterial substances by moulds is being carried out in this laboratory and has already led to the identification of a number of mould metabolic products and related substances which have striking antibacterial properties [Raistrick & Smith, 1941; Oxford, Raistrick & Smith, 1942; Oxford, 1942 *a, b*; Oxford & Raistrick, 1942]. In the course of this investigation it was noticed that a new species of *Penicillium*, *P. resticulosum* sp.nov., when grown on Czapek-Dox 5 % glucose solution, gives metabolism solutions which suppress completely the growth of *Staphylococcus aureus* in bullock's heart broth containing 2 % of glucose at dilutions of 1 : 320.

This antibacterial substance is very sensitive towards acids so that metabolism solutions from *P. resticulosum*, showing full activity at dilutions of 1 : 320, become entirely inactive on keeping overnight at pH 2.0 or below. Ether extraction of such inactivated metabolism solutions led to the isolation, in considerable yield, of a new mould metabolic product of Structure I. This substance, which may be regarded as a simple peptide of fumaric acid and *dl*-alanine, has, for convenience, been named *fumaryl-*dl*-alanine* in accordance with the ordinary peptide nomenclature although this name is not strictly correct, a more accurate designation being *fumaromono-*dl*-alanide*.

The intriguing possibility therefore arose that this compound forms an integral part of the molecule of the antibacterial substance produced by this mould. Unfortunately such evidence as we have been able to adduce up to the present, while not absolutely conclusive, tends to disprove this possibility. Fumaryl-*dl*-alanine is a strongly acidic substance and hence cannot be extracted with ether from active metabolism solutions (pH 4.1) without prior acidification to pH 2 or below, a proceeding which itself inactivates the antibacterial substance. However, we have found that the antibacterial substance is precipitated in a crude but active form by addition of acetone to the mould metabolism solution. This active precipitate is completely inactivated on treatment with cold *N*/10 HCl but no fumaryl-*dl*-alanine was obtained on ether extraction of the inactivated solution.

From what is known at present of the properties of the antibacterial substance from *P. resticulosum* it is clear that it is not penicillin.

The molecular constitution of the new mould metabolic product as fumaryl-*dl*-alanine was established as follows. The substance crystallizes in colourless needles, m.p. 229° (decomp.) having the empirical formula $\text{C}_7\text{H}_9\text{O}_5\text{N}$ and titrates as a dibasic acid. It contains no amino-acid nitrogen liberated on treatment with nitrous acid. Hydrolysis by boiling with 2*N* H_2SO_4 for 17 hr. converts all the nitrogen into amino-acid nitrogen and the products of hydrolysis, isolated in approximately equimolecular proportions, are fumaric acid and *dl*-alanine.

The fumaric acid was characterized by analysis, titration and mixed m.p. with an authentic specimen. Its identity was confirmed by the preparation of its characteristic reaction product with diazomethane, i.e. dimethyl-4:5-pyrazolinedicarboxylate [v. Pechmann, 1894], m.p. and mixed m.p. with an authentic specimen, 96–97°.

The *dl*-alanine was characterized by analysis and by comparison of the natural product and of its picrolonate with authentic synthetic *dl*-alanine and its picrolonate.

The mould metabolic product, on catalytic reduction with palladium-charcoal-hydrogen, gives *succinyl-dl-alanine*, colourless needles, m.p. 157°.

The molecular constitution was confirmed by synthesis. Synthetic *dl*-alanine (1 mol.) was treated in dry ether with fumaryl chloride (1 mol.), $\text{Cl} \cdot \text{CO} \cdot \text{CH} = \text{CH} \cdot \text{CO} \cdot \text{Cl}$. The resulting condensation product,

on treatment with water, gave fumaryl-*dl*-alanine identical in its properties with the natural product.

We have been at some pains to establish beyond reasonable doubt the fact that the mould metabolic product is optically inactive and that the amino-acid hydrolysis product is indeed *dl*-alanine and not one of the optically active forms. This is a matter of some importance in view of the rare occurrence in nature of *dl*-amino-acids though, in this connexion, it may be significant that *dl*-alanine has been previously recorded as a fungal metabolic product. Winterstein, Reuter & Korolew [1913] isolated *dl*-alanine from an aqueous extract of the higher fungus *Boletus edulis*. Our evidence is the following: (a) A solution of the natural product in ethanol, equivalent to 6.63 g. in 100 g. of solvent gave no measurable rotation in a 0.5 dm. tube. (b) A 5% solution in 2*N* H_2SO_4 after complete hydrolysis gave no measurable rotation in a 4 dm. tube. (c) An aqueous solution of the hydrochloride of the alanine isolated from the hydrolysed metabolic product (*c* = approx. 10%) gave no measurable rotation in a 0.5 dm. tube. Fischer [1906] gives the rotation of *d*-alanine hydrochloride as $[\alpha]_D^{20} = +10.4^\circ$ (*c* = 10% in water).

EXPERIMENTAL

Culture

The culture used throughout this work was *Penicillium resticulosum* sp.nov., L.S.H.T.M. catalogue no. P 47. Since this species is a new one and has not been described previously a full diagnosis is given.

Diagnosis

Penicillium resticulosum sp.nov. Coloniis in agar Czapekii late crescentibus, raris, primo albis, dein subglaucis cum marginibus funiculosus latissimis, denique aequaliter griseo-viridibus, floccosis et funiculosis, late zonatis in cultis senibus; guttulis crebris, minutis, subvinosis; reverso celeriter castaneo in media parte, succineo in margine, tarde subzonato; conidiophoris longis, funiculosis non vere fasciculatis, glabris, 3–4 μ diam.; penicillis compactis, bi- trive-verticillatis, plerumque cum axe principale, metulas sterigmatesque gerente, et uno duobusve ramis secundariis confertis ex nodis inferioribus, ad 100 μ long.; metulis 14–19 $\times$ 3.3–5 μ , subclavatis aut cum apicibus inflatis; sterigmatibus 9–10 $\times$ 2.2–3 μ ; conidiis primo citriformibus catenis oidiorum similibus, deinde subglobosis, glabris, 3–4 $\times$ 2.8–3.2 μ , in catenis primo parallelis demum implicatis.

Coloniis in musto ex hordeo cum agar in tenebris diu sterilibus, in luce conidia celeriter gerentibus.

Fungus alium cultum contaminans inventus.

Colonies on Czapek agar spreading broadly, tenuous, at first white then pale bluish green with very broad funiculose margin, becoming fairly dark grey-green all over, floccose funiculose, especially in outer areas, broadly and indistinctly zoned in old cultures; drops numerous during the growing period, small, pinkish; reverse quickly reddish brown to chestnut brown, shading to dull yellow near the margin; conidiophores long, mostly loosely aggregated in ropes rather than in true fascicles, smooth, $3-4\mu$ in diam.; penicilli very compact, 2- to 3-verticillate, often with main axis bearing a terminal verticil of metulae and sterigmata and one or two appressed secondary branches bearing sterigmata at approximately the same level as the main axis, up to 100μ in total length; metulae somewhat clavate or definitely swollen at the tip, $14-19 \times 3-3.5\mu$ (4.5μ at tip); sterigmata $9-10 \times 2.2-3\mu$, widening upwards then tapering abruptly to fine points; conidia cut off as more or less citriform segments, often resembling chains of oidia, subglobose when ripe, smooth, $3-4 \times 2.8-3.2\mu$; conidial chains at first roughly parallel, in old cultures becoming much tangled.

Cultures on wort agar remain sterile for several weeks if kept in the dark but well established colonies may be induced to form abundant spores by exposure for a few hours to diffuse light.

Related to *P. terrestre* Jensen and *P. solitum* Westling, but differing from both in texture of colonies and in colour.

Found as a culture contaminant in the laboratory. The name chosen for the new species is descriptive of the colony texture. (Latin *resticula* = a thin rope or cord.)

Cultural conditions

The culture medium used was a Czapek-Dox solution of the following composition: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1000 ml.; initial pH, 4.3. This medium was distributed in 350 ml. amounts in each of 100 one-litre conical flasks plugged with cotton wool. Fifty of the flasks were sterilized without further treatment (batch I); 1 ml. *N* NaOH was added to each of the remaining 50 flasks before sterilization, thus raising the initial pH to 6.0 (batch II). After sterilization, all flasks were sown with a spore suspension of *P. resticulosum*, P 47, prepared from cultures grown for 2 weeks on beer-wort agar and were then incubated in the dark at 24° .

The mould quickly formed a substantial felt, white at first, gradually developing patches of tan and purplish brown on a dirty white background, with edges showing streaks of brownish purple and reverse of felt yellowish tan. Sporing was spasmodic and was usually delayed until the cultures were about 3 weeks old, when a proportion of the flasks began to show floccose patches of greyish blue-green, particularly evident round the edges of the felt. The culture medium rapidly became dull yellow, darkening only a little on continued incubation. Filtered metabolism solutions, however, rapidly darkened to brownish orange on standing. There was no obvious difference in appearance between batches I and II.

Isolation and purification of fumaryl-dl-alanine

At the end of the incubation period the metabolism solution from batch I was filtered from the mycelium which was washed well with distilled water. The filtrate and washings were evaporated in vacuo to about 400 ml. and the concentrate was acidified by the addition of 5*N* HCl (100 ml.). The acidified liquid was shaken with ether (1 litre), the acid extracted by the ether then being transferred to saturated aqueous NaHCO_3 (20 ml.). The ether was now used again, without further treatment, to re-extract the acidified metabolism solution, the whole process being repeated 10 times. After the first two extractions the amount of NaHCO_3 used was reduced to 10 ml. each time. The combined NaHCO_3 extracts were acidified with HCl and subjected to continuous extraction with

ether. The solid crystallizing from the ether each day was removed, the extraction being continued for four working days. A total of 2.91 g. of crystals, m.p. 226°, was thus obtained. Evaporation of the ether mother liquors yielded a further 0.42 g. of slightly less pure crystalline material and finally an oily residue, wt. 4.05 g. Total yield of crude fumaryl-*dl*-alanine from 50 flasks of batch I=3.33 g.

Fifty flasks of batch II, on similar treatment, gave 3.96 g. of crude fumaryl-*dl*-alanine and an oily residue.

Experimental details of batches I and II are summarized in Table I.

Table I

Batch	Incubation period days	Initial pH	Final pH	Residual glucose by polarimeter %	Yield of crude product g.
I	19	4.3	4.1	0.81	3.33
II	20	6.0	4.1	0.80	3.96

The crude crystalline fumaryl-*dl*-alanine, m.p. 226°, obtained from both batches was already fairly pure. On recrystallization from glacial acetic acid with the addition of a little decolorizing carbon the m.p. was raised to 228°. Finally, recrystallization from water gave a product with a constant m.p. of 229° (decomp.).

*General properties of fumaryl-*dl*-alanine*

The substance crystallizes in colourless needles, m.p. 229° (decomp.). It is readily soluble in ethanol, hot water (cold water, 2–3%), hot glacial acetic acid (cold, about 1%), slightly soluble in ether, insoluble in light petroleum. It is a strong acid and is soluble in aqueous NaHCO₃ or Na₂CO₃ with the liberation of CO₂. It gives a strong fluorescein reaction when tested according to Feigl [1939, p. 299] for 1:2-dicarboxylic acids, probably due to hydrolysis to fumaric acid and alanine during the heating with conc. H₂SO₄ and resorcinol.

It is optically inactive. 0.0512 g. dissolved in 0.7718 g. of ethanol ($\equiv$ 6.63 g. in 100 g. of ethanol) gave no rotation in a 0.5 dm. tube in the mercury green light, $\lambda=5461$. (Found: C, 44.80; H, 4.98; N, 7.42%; CH₃O, nil; equiv. by titration, 94.5. C₇H₅O₅N requires C, 44.92; H, 4.85; N, 7.49%; equiv. titrating as a dibasic acid, 93.6.)

*Derivatives of fumaryl-*dl*-alanine*

(a) *Reaction product with diazomethane.* The metabolic product (0.1 g.) suspended in ether (5 ml.) was treated with an excess of diazomethane in ether. Brisk evolution of nitrogen occurred and the metabolic product dissolved. Later the reaction product separated in crystalline form and was collected. Wt. 0.088 g.; m.p. 162–165°. It was purified by recrystallization from ether followed by sublimation in a high vacuum at 115°. Colourless micro-crystals which on heating sintered above 160°, melted at 164–167° when tiny bubbles were evolved and the liquid cleared at 169°. It reset at 152° to a pasty crystalline mass which remelted at 169°, again evolving bubbles. There was no appreciable darkening. (Found: C, 46.96, 46.95; H, 5.81, 5.86; N, 15.8; CH₃O, 23.3%. C₁₀H₁₅O₅N₃ requires C, 46.69; H, 5.88; N, 16.3; 2CH₃O, 24.1%.) This reaction product is obviously derived by the methylation of two COOH groups and the addition of CH₂N₂, in a manner analogous to the reaction of fumaric acid with diazomethane, see p. 833.

(b) *Catalytic reduction product. Succinyl-*dl*-alanine.* The metabolic product (0.43 g.) dissolved in ethanol (12 ml.) was reduced with a palladium-charcoal catalyst (from 0.1 g. of palladium chloride) in an atmosphere of hydrogen. Observed uptake of hydrogen, 49.8 ml. (corr.); calc. for uptake of 2H per mol., 51.5 ml. The catalyst was separated by filtration and on removal of the solvent in vacuo a colourless crystalline solid remained (wt. 0.44 g.; m.p. 145° to a cloudy melt clearing at 150°). The crude product was crystal-

lized from glacial acetic acid and *succinyl-dl-alanine* was thus obtained as colourless needles, m.p. 157°, which are readily soluble in water and ethanol, slightly soluble in ether and insoluble in light petroleum. (Found: C, 44.69; H, 5.84; N, 7.45%. $C_7H_{11}O_5N$ requires C, 44.44; H, 5.86; N, 7.40%.)

Acid hydrolysis of fumaryl-dl-alanine

A mixture of the metabolic product (2.037 g.) and 2N H_2SO_4 (40 ml.) was boiled under reflux. The metabolic product quickly dissolved. At intervals the solution was cooled and 0.2 ml. was withdrawn for an amino-acid nitrogen estimation by the Van Slyke method. The amino-acid nitrogen found after various intervals was as follows: 0 hr., nil; 2 hr., 0.219 mg.; 5 hr., 0.560 mg.; 9½ hr., 0.643 mg.; 13½ hr., 0.707 mg.; 17 hr., 0.753 mg.; calc. for conversion of all nitrogen present to amino-acid nitrogen, 0.763 mg. After 17 hr. the hydrolysis was stopped and, on cooling, a colourless crystalline substance (A) separated. The clear supernatant liquid (B) showed no rotation in a 4 dm. tube.

Isolation and characterization of fumaric acid. The colourless crystalline substance (A) was separated by filtration, washed with water and dried. Wt., 0.87 g.; m.p. (in a sealed tube) 286°, alone or in admixture with authentic fumaric acid. The supernatant liquid (B) and washings, on continuous extraction with ether, yielded 0.30 g. of slightly impure fumaric acid. Total yield, 1.17 g.; calc. 1.26 g. The fumaric acid was purified for analysis by sublimation in a high vacuum. (Found: C, 41.43; H, 3.52%; equiv. by titration, 58.3. $C_4H_4O_4$ requires C, 41.39; H, 3.47%; equiv. titrating as a dibasic acid, 58.0.)

The identity of the fumaric acid was confirmed by conversion into the characteristic dimethyl-4:5-pyrazolinedicarboxylate [v. Pechmann, 1894]. The acid (0.1 g.) was suspended in ether (15 ml.) and treated with an excess of diazomethane in ether. When the vigorous reaction was complete, part of the ether was removed. On standing overnight the solution deposited a lustrous, colourless mass of plates, m.p. 94–95°, raised to 96–97° by sublimation in a high vacuum and unchanged on admixture with dimethyl-4:5-pyrazolinedicarboxylate prepared by the same method from authentic fumaric acid. (Found: C, 45.35; H, 5.56; N, 15.4; CH_3O , 33.2%. $C_7H_{10}O_4N_2$ requires C, 45.16; H, 5.41; N, 15.05; $2CH_3O$, 33.3%.)

Isolation and characterization of dl-alanine. The supernatant liquid (B) and washings, after ether extraction, were quantitatively freed from H_2SO_4 by the addition of $Ba(OH)_2$. The filtrate was concentrated in vacuo and finally dried over conc. H_2SO_4 to give a colourless crystalline residue. Wt., 0.67 g.; calc., 0.97 g.; m.p. 276°. (Note that the calculated theoretical yields of fumaric acid and alanine are not corrected for the amounts of hydrolysis solution used for amino-acid nitrogen estimations.) Recrystallization of the crude product from hot water (10 ml.) + hot ethanol (40 ml.) yielded 0.38 g. of rods or blunt-ended needles, m.p. 290° (decomp.) and a second crop, wt., 0.12 g., m.p. 280° (decomp.). On sublimation in a high vacuum at 180°, micro-crystals (needles) were obtained, m.p. 298° (decomp.) alone or in admixture with sublimed, synthetic, *dl*-alanine. (Found: C, 40.65; H, 8.00; N, 15.5%. $C_3H_7O_2N$ requires C, 40.43; H, 7.92; N, 15.7%.)

The amino-acid had no optical rotation. 0.050 g., dissolved in 2N HCl (0.28 ml.) and diluted with water to a total weight of 0.5362 g., showed no rotation in a 0.5 dm. tube. Mean of several readings (a) mercury green light, $\lambda = 5461$, -0.001° ; (b) mercury yellow light, $\lambda = 5790$, -0.002° .

The identity of the *dl*-alanine from the mould metabolic product was confirmed by preparation of the picrolonate. The amino-acid (0.1 g.) and picrolonic acid (0.3 g.) were dissolved in hot water (3.5 ml.), filtered and allowed to cool. The yellow needles thus obtained were collected and washed with a little water. Wt., 0.32 g.; m.p. 214° (decomp.). After grinding the dry crystals with ether the m.p. was raised to 216–217° (decomp.) alone or in admixture with the picrolonate of synthetic *dl*-alanine. (Found: C, 43.75; H, 4.50; N, 19.1%. $C_{13}H_{15}O_7N_5$ requires C, 44.19; H, 4.28; N, 19.8%.)

Synthesis of fumaryl-dl-alanine. (Structure I)

This synthesis was carried out following the method of Anschütz [1890] for the preparation of fumaranilic acid. Synthetic *dl*-alanine (0.5 g.) was covered with dry ether (5 ml.) and treated with 0.3 ml. (= 1 mol.) of fumaryl chloride. The mixture was shaken occasionally. The ether soon became bright yellow in colour. After 6 hr. the ethereal solution was filtered from the insoluble matter (probably alanine hydrochloride) which was washed with dry ether. The solvent was removed and the solid residue was dried in a good vacuum over solid KOH to remove traces of fumaryl chloride. Wt., 0.41 g.; m.p. 194° (decomp.). On treatment with water (20 ml.) the condensation product (Structure II of introduction) slowly dissolved. The aqueous solution was subjected to continuous ether extraction and yielded a product (0.35 g.) which was refluxed with ether (15 ml.). The residual crystalline mass (0.20 g.) was recrystallized from glacial acetic acid (3 ml.) and fumaryl-*dl*-alanine (0.12 g.) was thus obtained as colourless needles, m.p. 229° (decomp.), not depressed on admixture with the mould metabolic product, m.p. 229° (decomp.). (Found: C, 44.70; H, 4.97; N, 7.0%. $C_7H_9O_5N$ requires C, 44.92; H, 4.85; N, 7.5%.)

Antibacterial properties of the metabolism solution of P. resticulosum

100 flasks of Czapek-Dox solution (initial pH 4.3) were prepared, sown with *P. resticulosum* and incubated exactly as described for batch I (see p. 831). They were tested at intervals for antibacterial activity by the method used here originally by Clutterbuck, Lovell & Raistrick [1932] for the assay of solutions of penicillin and later by Raistrick & Smith [1941] and by Oxford [1942 a] for the assay of other mould metabolic products showing antibacterial activity, e.g. citrinin and penicillic acid.

Serial dilutions of the filtered mould metabolism solution were made from 1 : 10 to 1 : 5120 in 5 ml. amounts of sterile broth (bullock's heart broth containing 0.5% NaCl, 1% Witte's peptone and 2% glucose; final pH, after sterilizing, 7.2). Each tube was inoculated with one standard loopful of a 24 hr. culture of *Staphylococcus aureus*, L.S.H.T.M. strain, in the same medium. The inoculated tubes were incubated at 37° and the results were read after 24 hr. The results obtained are summarized in Table 2.

Table 2

Incubation period days	Number of flasks	Glucose by polarimeter %	pH	Inhibition against <i>Staphylococcus aureus</i>	
				Complete	Partial
14	4	1.53	3.8	1 : 320	—
15	50	1.26	3.85	1 : 320	1 : 2560
21	1	0.53	4.0	1 : 320	1 : 2560
23	45	0.46	4.3	1 : 200	1 : 400

The figures in the second column give the number of flasks which were taken off and of which the contents were mixed before filtration and assay.

Similar tests carried out on batch I (initial pH 4.3; 19 days' incubation) and batch II (initial pH 6.0; 20 days' incubation) (see Table 1) gave complete inhibition of growth in both cases at dilutions of 1 : 320 after 24 hr. incubation of the inoculated broth cultures at 37°.

The marked susceptibility of this antibacterial substance to acid was demonstrated as follows. 10 ml. of the average sample from 50 flasks taken off after 15 days' incubation (see Table 2) were acidified with 1 ml. *N* HCl, held at room temperature overnight and then neutralized with 1 ml. *N* NaOH. At the same time a control was set up in which 2 ml. *N* NaCl were added to another 10 ml. of the same sample after standing overnight. Antibacterial tests showed full growth of *Staphylococcus aureus* at all dilutions of the acidified sample but complete inhibition of growth at 1 : 320 and partial inhibition at 1 : 1280 in the control.

Isolation and general properties of the crude antibacterial substance

The antibacterial substance present in the metabolism solution of *P. resticulosum* has not yet been obtained pure. Hence only the results of preliminary experiments will be given here, fuller details being reserved for a later communication.

The filtered metabolism solution from 100 flask cultures grown on Czapek-Dox solution (initial pH 4.3) and giving total inhibition of the growth of *Staphylococcus aureus* at a dilution of 1 : 80 was evaporated in vacuo (bath temperature 40–45°) to about 1/7 of its volume. During this process about one-half of the original antibacterial activity was lost. An equal volume of acetone was added to the concentrate and after standing overnight at 0° the precipitate was separated by centrifuging. The supernatant aqueous acetone solution, after removal of the acetone, contained very little antibacterial substance. The acetone precipitate was extracted with cold distilled water (100 ml.), re-centrifuged and re-extracted with 100 ml. of water. The combined aqueous extracts (200 ml.) were treated with acetone (400 ml.) and the resulting precipitate was separated by centrifuging and dried in vacuo. Wt. 3.87 g.

The crude antibacterial substance thus obtained has the following properties. It resembles a pale brownish-yellow resin which can be easily powdered and it is readily soluble in water. It contains nitrogen, 3.8 % of ash and gives a positive Molisch reaction. It completely inhibits the growth of *Staphylococcus aureus* in glucose broth at a concentration of 1 : 160,000 with partial inhibition of growth at 1 : 320,000. A 1 % solution in *N*/10 HCl is completely inactivated on standing overnight at room temperature. A solution of the substance (1 g.) in *N* HCl (50 ml.) yielded only a trace (about 5 mg.) of extract on prolonged extraction with ether. This extract could not be identified as fumaryl-*dl*-alanine. If the scission of fumaryl-*dl*-alanine from the antibacterial substance on treatment with dilute acid were in fact the cause of the loss of antibacterial activity its presence would almost certainly have been revealed by the above procedure (see p. 831).

SUMMARY

Penicillium resticulosum sp. nov., when grown on Czapek-Dox 5 % glucose solution, produces considerable quantities of the hitherto undescribed fumaryl-*dl*-alanine (fumaro-mono-*dl*-alanide); this substance has been synthesized. Metabolism solutions of *P. resticulosum* show considerable antibacterial activity and the antibacterial substance has been isolated in a crude form. Fumaryl-*dl*-alanine does not appear, however, to form an integral part of the molecule of the antibacterial substance.

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