PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF SYNNEMATIN[1]

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Received for publication August 25, 1958

The antibiotic synnematin B (Gottshall *et al.*, 1951; Olson *et al.*, 1953) was later found (Abraham *et al.*, 1955) to be identical to cephalosporin N, which is (D-4-amino-4-carboxy-*n*-butyl) penicillin (Newton and Abraham, 1954). Olson *et al.* (1954) have reported production of 400 units of synnematin B per ml by *Cephalosporium salmosynnematum* growing on a corn meal-soybean meal medium, and isolation of the antibiotic at 75 per cent purity. Abraham *et al.* (1954) prepared cephalosporin N of 80 per cent purity, and Clark *et al.* (1957) and Fusari and Machamer (1958) have recently reported purification procedures for obtaining about 85 per cent pure synnematin B.

The presence of the perfect stage in the life cycle of Cephalosporia together with other morphological similarities have indicated that *C. salmosynnematum* belongs to the genus *Emericellopsis* in the family *Aspergillaceae* (Glosklags and Swift, 1957). It was found that several members of the genus *Emericellopsis* produce antibiotics similar to synnematin.

A synthetic medium for synnematin production with *Emericellopsis terricola* var. *glabra* was reported by Bhuyan and Johnson (1958). Synnematin labeled with S^{35} was isolated from a *C. salmosynnematum* or *E. terricola* var. *glabra* fermentation on a natural medium (Bhuyan *et al.*, 1958). It was also reported that the S^{35}-synnematin was purified by the use of a charcoal elution column and a Celite partition column. Recently Harvey and Olson (1958) have described chemically defined media for synnematin production.

The work reported here was undertaken to develop a natural medium for synnematin production by *E. terricola*, to isolate pure synnematin B, and to definitely determine the identity of the antibiotic.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from Wyeth Laboratories, West Chester, Pennsylvania.

EXPERIMENTAL METHODS

Fermentation techniques. E. terricola var. *glabra* (obtained from the Department of Botany, University of Wisconsin) was used exclusively in these experiments. The culture was maintained on soil in the manner described by Backus and Stauffer (1955). Spores of *E. terricola* were grown on honey peptone medium, as described by Bhuyan and Johnson (1958). Ten ml of sterile water were dispensed into 6-ounce spore bottles. Five ml of the resulting spore suspension were used to seed 50 ml of the spore germination medium in 500-ml Erlenmeyer flasks, which contained (per L): soybean meal, 20 g; corn meal, 20 g; and $CaCO_3$, 5 g. These flasks were shaken for 48 hr, and 5 ml of the germination medium were used to seed 50 ml of the same medium in 500-ml Erlenmeyer flasks. These flasks were shaken for 24 hr, and 2.5 ml of the vegetative growth were used to inoculate 50 ml of the fermentation medium in 500-ml, cotton-plugged Erlenmeyer flasks. The salt mixture used in all experiments consisted of (per L): $MgSO_4 \cdot 7H_2O$, 2.5 g; KH_2PO_4, 30.0 g; Na_2SO_4, 5.0 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1.0 g; $CuSO_4 \cdot 5H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $MnSO_4 \cdot H_2O$, 0.2 g. After the medium was autoclaved, it was adjusted with sulfuric acid to about pH 6. Adjuvant amino acids (as hydrochlorides) were dissolved in water, adjusted with sodium hydroxide to pH 6.0, and autoclaved. All fermentations were run at 30 C on a Gump rotary shaker, which operated at 250 rpm and described a 2¼ in circle. All fermentations were conducted in duplicate and the figures reported here are the averages of these two flasks. Samples (2 ml) were removed at intervals for analyses.

Analytical procedures for fermentation. Synnematin was assayed by the Oxford cup method with *Micrococcus pyogenes* var. *aureus* or *Bacillus subtilis* as the test organism and synnematin B (prepared by Abbott Laboratories, North Chi-

cago, Illinois, and Division of Laboratories, Michigan Department of Health, Lansing, Michigan) as the standard. Because the only standard available contained more than one antibiotic, the potency obtained for even a homogeneous unknown sample is not independent of assay procedure. Assay figures were therefore regarded as relative, not absolute, indications of yield or purity. The pH was determined with a glass electrode immediately after removal of the sample. All sugars were determined by the method of Shaffer and Somogyi (1933). The method for determination of ammonia nitrogen was that described by Gailey *et al.* (1946). Soluble Kjeldahl nitrogen was determined by the method of Johnson (1941). The mycelial nitrogen was determined by subtracting the soluble nitrogen present at the time of sampling from the soluble nitrogen present at the time of inoculation.

Materials. Ground whole corn meal obtained from the University of Wisconsin farms was used. Corn steep liquor was obtained from the A. E. Staley Company, Decatur, Illinois. Soybean meal was obtained as soybean oil meal flakes from Archer-Daniels-Midland Company, Decatur, Illinois. Soybean meal extract was prepared by heating 100 g of soybean meal (ground flakes) for 15 min at 100 C, with stirring with 1500 ml of distilled water. After cooling, the mixture was filtered through two thicknesses of cheesecloth, the liquid portion being pressed out of the cloth. The yield was about 1 L of extract.

Purification procedures. The medium used for fermentation contained, per 50 ml: glucose, 2 g; $CaCO_3$, 0.5 g; soybean meal extract, 20 ml; $(NH_4)_2SO_4$, 0.05 g; salt mixture, 5 ml; and $S^{35}O_4$, 1.0 mc. At 12-hr intervals, beginning 24 hr after inoculation, 0.4 per cent of cottonseed oil and 0.04 per cent of L-lysine were added.

The culture fluid obtained after a 7- or 8-day fermentation (1000 to 1100 units per ml) was filtered and the mycelium washed with water. The broth and washings were adjusted to pH 6.3 with N H_2SO_4, and poured into a carbon elution column. The carbon column was prepared as follows: Darco G 60 (18 g), Celite 545 (54 g), and N HCl (450 ml) were mixed and shaken for 15 min. The mixture, after filtration, was washed with water, then with pH 6.3 phosphate buffer (1 per cent w/v) until the acid had been removed. The carbon and Celite filter cake was suspended in the phosphate buffer and poured into a column (3.2 by 27 cm). Subsequent adsorption and elution were run at 3 to 4 C. The broth and washings (24.1×10^4 units) were allowed to percolate through the column, followed by washing with 340 ml of water. After the washing, the synnematin was eluted with 60 per cent (v/v) acetone in water at a rate of 1 ml per min. The eluate was collected in 10-ml fractions. After demonstration of bioactivity and radioactivity of each fraction, active fractions were combined and the acetone evaporated in vacuum to aqueous solution, which was then lyophilized. The lyophilized product (565 mg) was dissolved in 5 ml of water, followed by addition of 38 ml of methanol. After removal of the resulting precipitate, a colorless supernatant was obtained, to which 140 ml of acetone were then added. The precipitate formed was filtered off, and dried in vacuum at room temperature. The product obtained was used for the next step, Celite 545 partition column chromatography.

The Celite 545 partition column was prepared as follows: 1600 ml of butanol, 1066 ml of 95 per cent ethanol, and 1600 ml of 5 per cent (w/v) aqueous citric acid (adjusted to pH 6.10 with solid KOH) were equilibrated at 3 to 4 C and the two phases separated. Organic phase (1250 ml), aqueous phase (50 ml), and 100 g of Celite 545, were then mixed together by shaking. The slurry was then poured into a column (3.4 by 40 cm). Excess organic phase was used for developing the column. Subsequent procedures were also carried out at 3 to 4 C. The synnematin preparation (270 mg) was dissolved in 4 ml of solvent phase, which was put on the column. A few drops of organic phase saturated with Sudan III were added to the synnematin sample to mark the solvent front. The column was then developed and 13.7-ml fractions were collected at the rate of 16 ml per hr. Each fraction was analyzed for bioactivity and radioactivity. The pooled active fractions were concentrated in vacuum to syrup at 35 C. The syrup was extracted with 50 ml of methanol, which was then evaporated to about 10 ml. Addition of 40 ml of acetone to the concentrate yielded a slight amount of precipitate. The supernatant was dried in vacuum. A product obtained weighed 53 mg and assayed 806 units per mg.

Analytical procedures for purification. Biological activity in effluent fractions was determined by the *B. subtilis* blotter disc bioassay described by Bhuyan *et al.* (1958). Radioactivity in effluent fractions was determined by a thin-window

Geiger counter. Corrections were made for coincidence, self-absorption, and background.

Synnematin chromatograms were made by the method used by Bhuyan *et al.* (1958). Bioautographs were made by placing the paper strips (after development with the solvent) on the agar medium seeded with spores of *B. subtilis*. Radioactive chromatograms were counted with a thin-window Geiger counter, connected with a count rate meter and a recorder, as described by Schepartz and Johnson (1956).

Determination of side chain of synnematin B. Synnematin B and penicillin G preparations were hydrolyzed with N HCl for 9 hr at 100 C. Amino acids liberated by hydrolysis were detected by paper chromatography as described by Clayton and Strong (1954). Ascending chromatograms were run with a solvent system consisting of 300 ml of methyl ethyl ketone, 100 ml of propionic acid, and 120 ml of water. The papers were developed for 20 hr in a 40-L jar at 25 C.

Isolation of D-*α-aminoadipic acid.* Synnematin B preparations (40 mg, 98.9 per cent purity) were hydrolyzed in 6 ml of N HCl for 7 hr at 100 C. The hydrolyzate was evaporated to dryness in a cold air stream. The residue was dissolved in 4 ml of water and adjusted to pH 4.1 with N NaOH. This material was chromatographed on a column (1.7 by 15 cm) of Amberlite IR-4B (50 mesh, previously treated with HCl, then washed with water until the pH of the effluent was between 3.5 and 4.0). After introduction of the sample, water was added to the column at the rate of 0.7 ml per min. Eluate was collected in 8.5-ml fractions. After 11 fractions of water effluent had been collected, N HCl was substituted for water. Development was stopped after 11 fractions of acid effluent had been collected.

The fractions containing the amino acids were located roughly by spotting 2-μL samples of each fraction on a sheet of filter paper and spraying with ninhydrin solution. Blue spots developed from fractions 3–7 of water effluent and 5–9 of acid effluent. The former and latter fractions were found by paper chromatography to contain β-thiovaline and α-aminoadipic acid, respectively. The fractions containing α-aminoadipic acid were concentrated to 4 ml in a cold air stream and adjusted to pH 3.2 with N NaOH. The yellow precipitate obtained was filtered off and washed with a few ml of water and alcohol. The vacuum-dried precipitate weighed 5 mg. A further 10 mg of precipitate were obtained by concentrating the mother liquor and acidifying to pH 3.2. The combined material was dissolved in 5 ml of hot water and 0.1 g carbon added to the solution (pH 3.7). After removal of the carbon cake the colorless solution obtained was concentrated to 3 ml and adjusted to pH 3.2 with 0.1 N HCl. It was then left to crystallize overnight. The crystals were filtered and washed with cold water. The first crop yielded 2 mg. A further 3 mg were obtained by concentrating the mother liquor and acidifying to pH 3.2.

RESULTS

Synnematin production with E. terricola. E. terricola var. *glabra* has been found (Bhuyan and Johnson, 1958) superior to *C. salmosynnematum* in the following respects: (a) higher yields of synnematin, (b) greater utilization of inorganic sulfur in formation of synnematin and consequently a higher specific activity of synnematin, and (c) fewer S^{35}-contaminants. Therefore, the utilization of various organic materials as nitrogen sources in the synnematin fermentation by *E. terricola* var. *glabra* was investigated. The yields of synnematin with the most complete media developed for the natural materials are shown in table 1. The highest yields of synnematin were obtained when soybean meal extract was used as the nitrogen source. When glucose was fed intermittently to this medium, 497 units per ml of synnematin were obtained. Furthermore, synnematin production increased to 852 units per ml when lard oil was intermittently added to the medium.

Effects of oil on synnematin production. As shown in table 1, intermittent addition of lard oil was found to stimulate the synnematin production greatly. Therefore, the effects of the intermittent addition of several different oils on synnematin production were studied. As shown in table 2, all of them tested were found to increase the yields of synnematin remarkably. Cottonseed oil was found to give the highest yields (888 units per ml). Based on these fermentation data, it was found that a medium containing 4 per cent glucose, 1 per cent $CaCO_3$, 40 per cent soybean meal extract, 0.1 per cent $(NH_4)_2SO_4$, and 10 per cent salt mixture, with the intermittent feeding of cottonseed oil, was optimum for synnematin production. Therefore,

TABLE 1

Synnematin yield on various natural materials with Emericellopsis terricola

Natural Material	Glucose	$(NH_4)_2SO_4$	Feeding*	Max Yield	pH Plateau†	Time for Max Yield
	%	%		*u/ml*		*hr*
Corn meal, 4%; soybean meal, 4%	0	0.1	Oil	208	8.1	71
Soybean meal extract, 40%‡	4	0.1	Oil	852	7.1	170
Soybean meal extract, 40%‡	4	0.1	Glucose	497	7.6	170
Corn steep liquor (initial pH 5.5), 4%	4	0.1	Oil	690	7.3	144
Corn steep liquor (initial pH 6.0), 4%	4	0.1	Oil	533	7.5	120
Tryptone, 2%	4	0.1	Oil	437	7.7	170
Cane molasses, 8%; yeast extract, 0.5%	0	0.6	Glucose	267	7.5	192
Peptone, 4%	4	0.1	Oil	135	8.2	120
Yeast extract, 0.5%	4	0.8	Oil	134	7.2	96
Malt extract, 4%	4	0.1	Oil	70	5.0	144
Blood meal, 0.5%	4	0.8	Oil	0	(no growth)	

All media contained 1 per cent $CaCO_3$ and 5 ml of the salt mixture per 50 ml of medium.

* Beginning 24 hr after inoculation, 0.36 per cent of lard oil or glucose was added every 12 hr (intermittent addition).

† The pH plateau is defined as the average pH of the medium during the synnematin synthesis phase.

‡ Twenty ml of extract per 50 ml of medium.

this medium was used in all subsequent experiments.

Effects of amino acids on synnematin production. Since it has been shown that lysine can be converted to α-aminoadipic acid (Rothstein and Miller, 1954) and can arise from diaminopimelic acid (Dewey and Work, 1952), DL-α-aminoadipic acid, DL-diaminopimelic acid, and L-lysine were added to the synnematin fermentation. As shown in table 3, L-lysine increased the synnematin yield. The chemical changes in the fermentation to which 0.08 per cent L-lysine was added every 24 hr, beginning 24 hr after inoculation, are shown in figure 1. The pH plateau of the fermentation to which L-lysine HCl was added was found to be lower than that of the control. Therefore, it could not be concluded that L-lysine was effective until a comparison was made with a control maintained at the same pH plateau as the fermentation to which L-lysine was added.

Optimal pH for synnematin formation. In order to compare the effectiveness of L-lysine and DL-lysine at the same pH plateau as the control, the optimal pH for the synnematin production was first determined. The pH of the media was adjusted at the end of 60 hr when the growth was essentially completed. The pH was maintained at different levels by adjustment with N H_2SO_4 or N NaOH at 12-hr intervals. As shown in table 4, the optimum pH for synnematin production was found to be between 6.0 and 6.5 in a natural medium.

TABLE 2

Effect of oil addition on synnematin fermentation with Emericellopsis terricola

Added Oil	Yield	pH Plateau	Time for Max Yield
	u/ml		*hr*
No addition*	451	7.4	120
Cottonseed oil	888	7.3	120
Soybean oil	876	7.0	170
Lard oil	852	7.1	170
Corn oil	768	7.3	120
Sesame oil	727	7.1	170
Linseed oil	664	7.2	120
Castor oil	609	7.2	170

All media contained glucose, 2 g; $CaCO_3$, 0.5 g; soybean meal extract, 20 ml; $(NH_4)_2SO_4$, 0.05 g; and 5 ml of salt mixture per 50 ml of medium.

Oil was added at the rate of 0.36 per cent every 12 hr, beginning 24 hr after inoculation.

The pH plateau is defined as the average pH of the medium during the synnematin synthesis phase.

* Glucose was added at the rate of 0.36 per cent every 12 hr, beginning 24 hr after inoculation.

Effects of L-lysine and DL-lysine on synnematin fermentation. A comparison was made with a control maintained at the same pH plateau as

TABLE 3

Effects of amino acids on synnematin fermentation with Emericellopsis terricola

Precursors Used	Rate of Addition	Started at	Yield	pH Plateau	Time for Max Yield
	%/24 hr	*hr*	*u/ml*		*hr*
L-Lysine	0.08	24	1201	7.00	168
L-Lysine	0.04	24	1061	7.06	144
L-Lysine	0.02	24	973	7.20	144
L-Lysine	0.08	48	1046	7.00	168
L-Lysine	0.04	48	1050	7.12	168
L-Lysine	0.02	48	1127	7.02	144
DL-Diamino-pimelic acid	0.04	48	899	7.39	144
DL-α-Amino-adipic acid	0.04	48	882	7.41	168
No addition			810	7.29	168

All media contained glucose, 2 g; $CaCO_3$, 0.5 g; soybean meal extract, 20 ml; $(NH_4)_2SO_4$, 0.05 g; and 5 ml of salt mixture per 50 ml of medium.

Cottonseed oil was added at the rate of 0.36 per cent every 12 hr, beginning 24 hr after inoculation. Amino acids were added at 12-hr intervals.

The pH plateau is defined as the average pH of the medium during the synnematin synthesis phase.

fermentations to which L-lysine or DL-lysine were added. The pH of the media to which 0.04 per cent L-lysine or 0.04 per cent DL-lysine were added was adjusted to pH 6.0 every 12 hr, beginning at the end of 60 hr, when the growth was essentially completed. The fermentation data in these experiments are listed in table 5. When L-lysine or DL-lysine was fed at the rate of 0.04 per cent per 12 hr, beginning 24 hr after inoculation, yields of 1015 units per ml for L-lysine or 1120 units per ml for DL-lysine were obtained, whereas the yields were 677 units per ml when no lysine was added. Therefore, it is clear from the results obtained here that L-lysine and DL-lysine stimulated synnematin production, probably acting as precursor.

In a charcoal elution column used for the first step of purification, a comparison was made of the elution pattern of the fermentation to which no L-lysine was added with that of the fermentation to which L-lysine was added. A comparison of figure 2(*a*) with figure 2(*b*) indicates that the elution pattern of the culture beer to which L-lysine was not added contained more radioactive contaminants, mostly around the synnematin peak, than that of the culture beer to which L-lysine was added. This difference suggests that addition of L-lysine as precursor results in more formation of synnematin B and less of radioactive contaminants.

Purification of S^{35}-synnematin B. In order to establish the chemical nature of the antibiotic produced by *E. terricola*, its purification was undertaken. Radioactive antibiotic was used because of the ease with which sulfur-containing contaminants could be detected. As shown in figure 3(*a*), a radioactive chromatogram of the culture beer shows three major peaks in addition to a peak at the origin ($-SO_4^-$). By comparison of the R_f values of the main inhibition zone of a known synnematin B bioautogram with those of a radioactive chromatogram and a bioautogram of S^{35}-synnematin preparations, the component

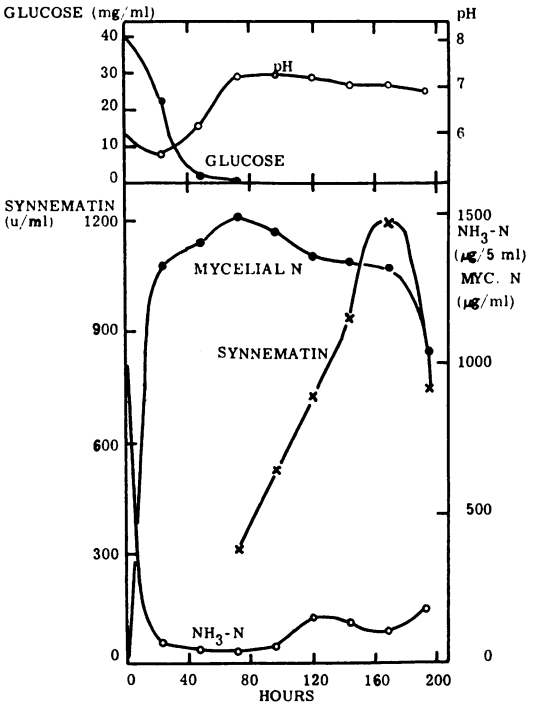

Figure 1. Chemical changes in a fermentation to which L-lysine was added. The medium contained 4 per cent glucose, 40 per cent soybean meal extract, 0.1 per cent ammonium sulfate, 1 per cent calcium carbonate, and 5 ml salt mixture per 50 ml of medium. Beginning at 24 hr, 0.08 per cent L-lysine was added every 24 hr. Beginning at 24 hr, 0.36 per cent cottonseed oil was added every 12 hr.

TABLE 4

Effect of pH on synnematin production

Average pH after 60 hr	Max Yield	Time to Max Yield
	u/ml	*hr*
5.74	425	192
6.05	814	168
6.17	827	192
6.63	594	168
7.08	470	144

The medium contained 4 per cent glucose, 1 per cent $CaCO_3$, 40 per cent soybean meal extract, 0.1 per cent ammonium sulfate, and 5 ml salt mixture per 50 ml of medium. Beginning at 24 hr, 0.4 per cent cottonseed oil was added every 12 hr. Beginning at 60 hr, the pH of the medium was maintained at different levels manually by adjustment with 1 N H_2SO_4 or 1 N NaOH at 12-hr intervals.

TABLE 5

Effect of L*-lysine and* DL*-lysine on Emericellopsis terricola fermentation*

Time	No Lysine Addition		L-Lysine Addition		DL-Lysine Addition	
hr	*pH*	*u/ml*	*pH*	*u/ml*	*pH*	*u/ml*
60	6.60		6.48		6.20	
72	6.98	219	7.23	247	7.18	304
84	6.17		6.31		6.21	
96	6.78	422	6.53	466	6.50	541
108	6.20		6.06		6.00	
120	6.25	495	6.08	664	6.25	709
132	6.05		6.05		6.06	
144	6.19	618	6.08	941	6.33	919
156	5.95		6.25		6.08	
168	6.23	677	6.38	1015	6.55	1120
180	5.93		6.00		6.15	
192	6.11	640	6.39	986	6.58	813
Avg pH...	6.13		6.16		6.18	
Max yield...		677		1015		1120

The medium contained 4 per cent glucose, 1 per cent $CaCO_3$, 40 per cent soybean meal extract, 0.1 per cent ammonium sulfate and 5 ml salt mixture per 50 ml of medium. Beginning at 24 hr, 0.4 per cent cottonseed oil was added every 12 hr. Beginning at 24 hr, 0.04 per cent lysine was added every 12 hr. Beginning at 60 hr, 1 N H_2SO_4 or 1 N NaOH was added every 12 hr to adjust the pH to 6.0.

at R_f 0.49 in figure 3(*a*) has been identified as synnematin B. Nothing definite is known about the component at R_f 0.85 except that it behaves as do nonpolar penicillins on this particular chromatogram. The peak at R_f 0.25 was previously reported by Bhuyan *et al* (1958) to be the penicilloic acid of synnematin B.

As the first step, a charcoal elution column was used for purification of synnematin B. Prelimi-

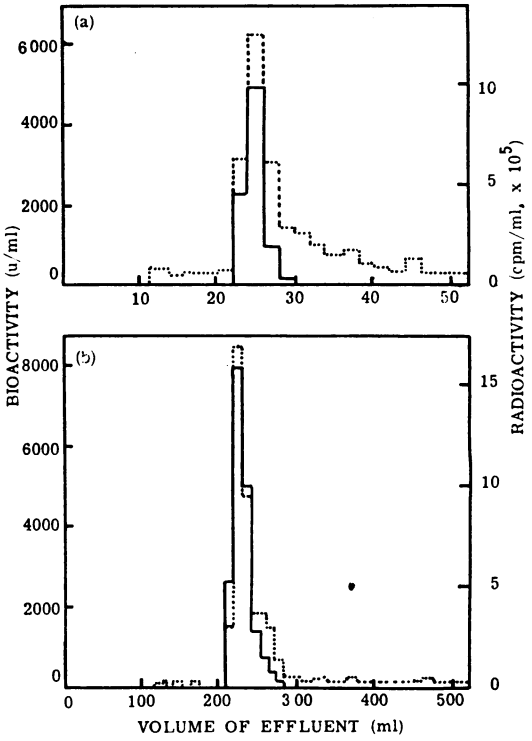

Figure 2. Charcoal purification of synnematin. The solid lines represent biological activity (units synnematin per ml) and the dotted lines represent radioactivity (counts per minute per ml; $\times 10^5$). (*a*) Charcoal elution pattern of the culture fluid of the fermentation to which no L-lysine was added as precursor. A total of 20,500 units of the culture filtrate were added to a column (1.7 by 10.0 cm) packed with 2 g of charcoal and 6 g of Celite 545, followed by elution with 60 per cent aqueous acetone. (*b*) Charcoal elution pattern of the culture fluid of the fermentation to which L-lysine was added as precursor. A total of 241,000 units of the culture filtrate were added to a column (3.2 by 26 cm) packed with 18 g of charcoal and 54 g of Celite 545, followed by elution with 60 per cent aqueous acetone.

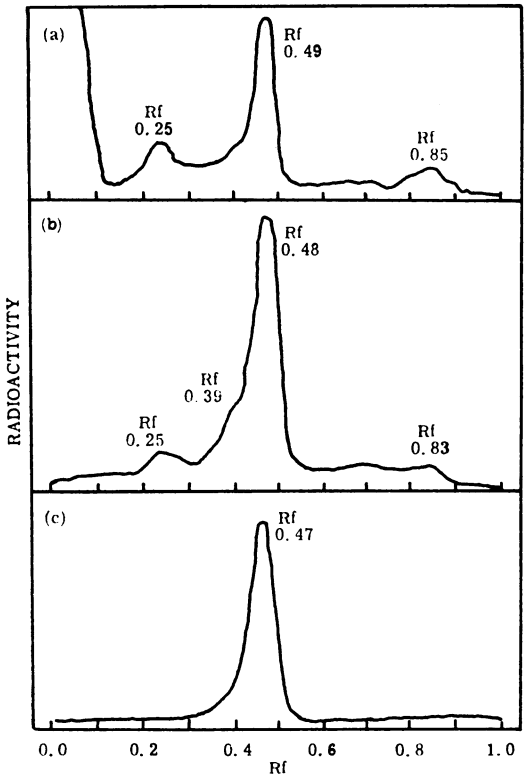

Figure 3. Radioactive chromatograms of S^{35}-synnematin at each stage of purification. (*a*) Filtered synnematin broth. (*b*) Biologically active effluent from the charcoal column. (*c*) Effluent from Celite 545 column at peak of biological activity. The chromatograms were made with Eaton-Dickeman 613 paper strips and the ethanol-butanol-water solvent system described under Methods. Recorder tracings of radioactivity on the strips were made as described by Schepartz and Johnson (1956).

nary experiments showed that approximately 26,000 units of synnematin could be adsorbed by 1 g of carbon. In the carbon column, the amounts of carbon used were about twice that necessary to adsorb the synnematin in the volume of beer used. The elution pattern of 24.1×10^4 units of culture beer is shown in figure 2(*b*). A radioactive chromatogram of the effluent at the synnematin peak in figure 2(*b*) is shown in figure 3(*b*). Radioactive contaminants are still present in the active effluent in the range of about one third of the total radioactive area. A small shoulder at R_f 0.39 may be a degradation product of synnematin, since it seems to accumulate during storage of synnematin. Synnematin preparations obtained by lyophilization gave about 250 to 300 units per mg. Further purification of the lyophilized product by the precipitation method as mentioned under Experimental Methods yielded a preparation of 350 to 400 units per mg, which was used for the next purification procedure.

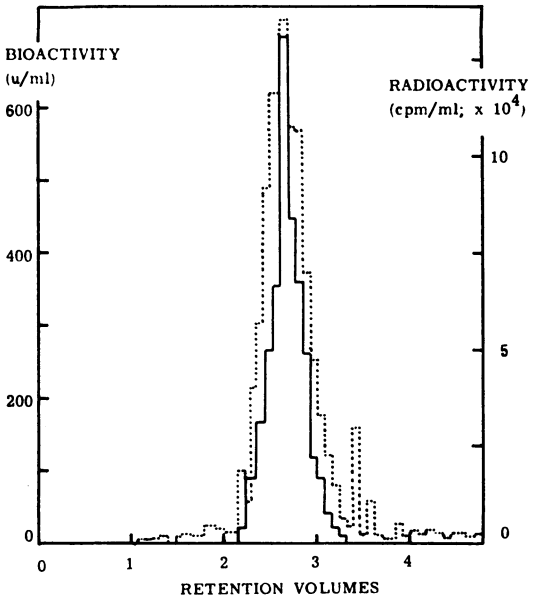

Figure 4. Celite 545 column chromatogram of synnematin B. The solid lines represent bioactivity (u per ml) and the dotted lines represent radioactivity (cpm per ml; $\times 10^4$). The sample put on this column was prepared by lyophilizing all biologically active fractions from a charcoal elution column, drying a precipitate obtained by addition of acetone to a methanol extract of the lyophilized product, and dissolving the product in 5 ml of organic phase of the present chromatographic system. Sudan III was added to mark the solvent front. The solvent system was prepared by equilibrating at 3 to 4 C, 1600 ml of *n*-butanol, 1066 ml of 95 per cent ethanol, and 1600 ml of 5 per cent aqueous citric acid adjusted to pH 6.10 with KOH. Column prepared from: Celite 545, 100 g; aqueous phase, 50 ml; organic phase, 1250 ml. Height of column, 40 cm; diameter of column, 3.4 cm; retention volume, 250 ml; flow rate, 4 ml per 15 min; retention volumes up to peak of synnematin bioactivity and radioactivity, 2.63; units of synnematin put on column, 9.5×10^4; recovery of biological activity, 70 per cent.

TABLE 6

Results of procedure for purification of S^{35}-synnematin

Preparation	Total Vol or Total Wt	Biological Activity		Radioactivity		Purity	Recovery
		u/ml or u/mg	Total units	cpm/ml or cpm/mg	Total cpm		
						%	%
Culture filtrate (diluted with water)	380 ml	634 u/ml	24.1×10^4				100
60% Acetone eluate from charcoal column	74 ml	3031 u/ml	22.4×10^4	7.16×10^5 cpm/ml	5.31×10^7		92.9
Lyophilized product	565 mg	256 u/mg	14.5×10^4	9.35×10^4 cpm/mg	5.28×10^7	30	60.2
Powder from acetone precipitation	270 mg	350 u/mg	9.5×10^4	1.24×10^5 cpm/mg	3.35×10^7	40	39.2
Eluate from Celite partition column	178 ml	373 u/ml	6.6×10^4	1.00×10^5 cpm/ml	1.78×10^7		27.5
Preparation from methanol extract	53 mg	806 u/mg	4.3×10^4	3.24×10^5 cpm/mg	1.72×10^7	95	17.7

Purity was determined by a chemical assay for penicillin G, using hydroxylamine. Recovery was determined by dividing total biological activity at every stage by that of the starting beer.

As the second step, Celite 545 partition column chromatography was adopted for further purification. Results of the column are given in figure 4. As shown in figure 3(*c*), a radioactive chromatogram of the effluent from the Celite column at the peak of bioactivity indicates that radioactive contaminants were completely removed from synnematin-containing fractions. Also, the R_f of the inhibition zone in bioautography of the synnematin fractions corresponded exactly to the R_f of the radioactive peak in the radioactive chromatogram. Therefore, S^{35}-synnematin B which is entirely free from S^{35}-contaminants has been prepared by means of Celite partition column chromatography. Further purification of the synnematin fractions, as mentioned under Methods, yielded 53 mg synnematin preparation of 806 units per mg. The specific activity of the product was 3.24×10^5 cpm per mg. By the hydroxylamine method of Ford (1947), with benzylpenicillin as a standard, this preparation (potassium salt) was found at assay to be 95 per cent pure. In this determination, it was assumed that equimolar quantities of penicillin and synnematin yield the same hydroxamate color intensity. The maximum purity thus far obtained in any run is 99.7 per cent. In an over-all purification procedure, starting from 24.1×10^4 units of culture beer, the biological activity, radioactivity, purity, and recovery at every purification stage are listed in table 6.

Determination of the side chain of synnematin B. Amino acids liberated by acid hydrolysis of synnematin preparations were detected by paper chromatography. As shown in table 7, the crude synnematin sample was found to contain glutamic, aspartic, α-aminoadipic acids and β-thio-

TABLE 7

R_f values obtained by paper chromatography of hydrolyzates of synnematin B preparations

	R_f values				
Synnematin B, 95% pure (2 spots)		0.12			0.26
Synnematin B, 30% pure (5 spots)	0.053	0.13	0.17	0.23	0.27
DL-α-Aminoadipic acid					0.26
β-Thiovaline from penicillin G		0.12			
L-Glutamic acid				0.23	
DL-Aspartic acid			0.16		
D-β-Thiovaline*		0.12			

The chromatographic systems are described under Methods. The 95 per cent pure synnematin B preparation was obtained by the procedure, in which a charcoal column and a Celite partition column were employed for its purification. The 30 per cent pure synnematin B preparation is a product obtained by lyophilization of effluent from charcoal column.

* D-Penicillamine HCl $\frac{1}{2}H_2O$, obtained from California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles, California.

valine, whereas the pure preparation showed the presence of α-aminoadipic acid and β-thiovaline. Newton and Abraham (1953) reported that traces of glutamic and aspartic acids were liberated from the purest preparation of cephalosporin N on acid hydrolysis at 100 C, and larger amounts from crude preparations. It is evident from the results obtained here that glutamic and aspartic acids are impurities in the crude sample.

Isolation of D-α-*aminoadipic acid from the hydrolyzate of synnematin B.* α-Aminoadipic acid was isolated in a crystalline form from the hydrolyzate of pure synnematin B, by using Amberlite IR-4B resin. The substance melted at 205 to 207 C; $[\alpha]_D^{20} = -24.2$ in 6 N HCl (c = 1.5 g per L); and the same R_f value as an authentic sample of DL-α-aminoadipic acid (obtained from H. M. Chemical Company, Ltd., Santa Monica, California) on a paper chromatogram. The mixed melting point with the authentic sample of DL-α-aminoadipic acid (mp 198) was 188 to 189 C. These data indicate that the R-group of synnematin B is D-α-aminoadipic acid.

DISCUSSION

An ideal medium, not only for synnematin but also for fermentation products secreted by microorganisms, should support a rapid rate of growth during the growth phase and a slow rate of growth during the product-secreting phase. Such requirements would account for remarkable effect of the slow addition of a utilizable oil on synnematin fermentation with *E. terricola*. The antifoam activity of the oil and maintenance of pH in a region suitable for synnematin production can be considered to be other reasons for the effectiveness of oil.

Most of the attempts to increase the yields were made by methods previously used in penicillin fermentation. Intermittent addition of glucose or oil at the rate of 0.36 to 0.40 per cent per 12 hr, beginning 24 hr after inoculation, has been used in penicillin fermentation (Soltero and Johnson, 1954; Anderson *et al.*, 1956). Although the rate of oil addition most suitable for synnematin fermentation was not determined, the same rate of oil addition as for penicillin fermentation was found to increase the yields of synnematin greatly. The optimum pH for synnematin production on a natural medium was found to be between 6.0 and 6.5, in good agreement with that on a synthetic medium (Bhuyan and Johnson, 1958).

Since the pH plateau of the fermentation to which L-lysine was added was found to be lower than that of the control, a comparison was made with a control maintained at the same pH plateau (6.0 to 6.5) as the fermentation to which L-lysine or DL-lysine was added. The effectiveness of L-lysine, and DL-lysine, probably acting as a precursor, was confirmed under such experimental conditions. Recent studies on inhibition of penicillin formation by amino acid analogues (Demain, 1957) showed that L-lysine HCl inhibited penicillin production. Such inhibition might be ascribed to synnematin formation, instead of penicillin production. Up to the present, attempts to produce synnematin B from *Penicillium chrysogenum*, by the addition of suitable precursors, have not been successful. However, it might be possible that synnematin B would be formed in a *P. chrysogenum* fermentation with intermittent addition of L-lysine and adjustment of the pH to the optimum for synnematin production.

SUMMARY

In an *Emericellopsis terricola* var. *glabra* fermentation, a medium containing 4 per cent glucose, 1 per cent $CaCO_3$, 40 per cent soybean meal extract, 0.1 per cent $(NH_4)_2SO_4$, and 5 ml of salt mixture per 50 ml was found to be suitable for synnematin production. Intermittent addition of one of several oils was found to stimulate the production of synnematin greatly. Highest yields (888 units per ml; roughly, 1 g per L) were obtained by feeding cottonseed oil to the soybean meal extract medium. When DL-α-aminoadipic acid and DL-diaminopimelic acid were tried as precursors for synnematin production, no significant increase in yield was observed with these compounds. When L-lysine was used, the yield of synnematin increased from 810 units per ml to 1201 units per ml. The optimal pH for synnematin production on the natural medium was found to be between 6.0 and 6.5. When L-lysine or DL-lysine was fed to the fermentation, 50 to 60 per cent increase in yield was obtained at the same pH plateau (6.0 to 6.5) as a control. Therefore, it was concluded that L-lysine and DL-lysine stimulated synnematin production, probably acting as precursor.

An attempt was made to purify S^{35}-synnematin

B produced when L-lysine was fed as precursor and 1.0 mc of $-S^{35}O_4$ added to each fermentation. A combination of a charcoal elution column and a Celite 545 partition column was successful in isolating S^{35}-synnematin B, entirely free from S^{35}-contaminants. Synnematin B preparations of more than 95 per cent purity (maximum purity, 99.7 per cent) were prepared in this way.

Acid hydrolysis of pure synnematin B liberated α-aminoadipic acid and β-thiovaline. α-Aminoadipic acid was isolated in a crystalline form.

It can be concluded that the antibiotic produced by *E. terricola* var. *glabra*, growing on a soybean meal extract medium, with intermittent feeding of cottonseed oil and L-lysine is identical with synnematin B.

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