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Improved Methods for Production, Isolation, and Assay of Two New Chloroisoxazoline Amino Acid Antitumor Antimetabolites: U-42,126 and U-43,795

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Improved fermentation and isolation procedures for antitumor antimetabolites U-42,126 and U-43,795 increased drug yields 30-fold. The sensitivity limit of a newly developed assay is 0.03 μg of U-42,126 and 2.0 μg of U-43,795 per ml. The in vitro antimicrobial effect of both drugs was antagonized by histidine.

Antitumor antibiotic U-42,126 (NSC-163501) was discovered by utilizing an in vitro screen designed for detection of antimetabolites (2). Its production by *Streptomyces sviceus* (3), isolation and structure determination as α *S*,5*S*-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (7), and biological activity (5) were described in a series of communications in 1972 and 1973. The antibiotic has the unique structure shown in Fig. 1. Recently a related amino acid, U-43,795 (NSC-176324) (Fig. 1), was isolated from the same fermentation producing U-42,126 and found to have hydroxylated structure α *S*,4*S*,5*R*-α-amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid (6). The presence of U-43,795 in mixtures with U-42,126 was originally obscured by the low antibacterial activity (8) of U-43,795 towards the organism used to monitor production and isolation. However, in spite of diminished antibacterial activity, the new amino acid had significant antitumor activity against L1210 lymphoid leukemia in mice (8).

The interesting antitumor activity of both U-42,126 and U-43,795 against murine leukemia L1210 necessitated the preparation of substantial amounts of each for evaluation in other tumor systems. Since previous technology (3, 7) was inadequate for this task, a considerable effort was spent to improve the microbiological production, to devise simplified and more efficient methods of extraction and separation of the agents, and to develop a highly sensitive microbiological assay.

We also investigated which of the naturally occurring metabolites can reverse (prevent) the inhibition of a sensitive microorganism by pure U-42,126. The results of all these studies are presented in this communication.

MATERIALS AND METHODS

Production in 5,000-liter fermentation tanks. (i) Primary vegetative inoculum. Primary vegetative inoculum of *S. sviceus* was produced in medium containing (per liter of distilled water): glucose, 10 g; peptone (Difco), 10 g; and yeast extract, 2.5 g. It was inoculated with vegetative inoculum stored in a liquid N_2 storage tank. The cultivation was done in 500-ml nonstippled flasks (with 100 ml of medium) on a rotary shaker (270 rpm) that were incubated for 72 h at 28 C.

(ii) Secondary vegetative inoculum. Primary vegetative inoculum (300 ml) was used to inoculate a 250-liter seed vessel containing (per liter) the following medium: cerelose, 10 g; steepwater, 10 g (Corn Products Co., Englewood Cliffs, N.J.); Pharmamedia, 2 g (Traders Oil Mill Co., Fort Worth, Tex.); liquid peptone, 10 g (Wilson Protein Technology, Calumet City, Ill.); and lard oil, 2 ml. This seed vessel was kept at a constant temperature of 28 C for 48 h.

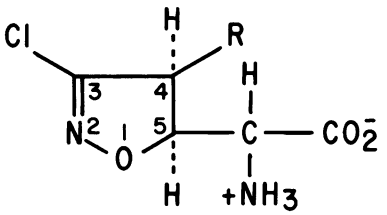
The air flow rate was 3.5 SCFM (standard ft^3/min of humidified air measured at 14.7 lb/in^2 and 70 F [ca. 21 C]) with 10 lb/in^2 gauge of tank pressure. The agitator speed was fixed at 280 rpm.

Fermentation. Fermentations were carried out in 5,000-liter tanks on a liquid volume of 4,000 liters in the fermentor after inoculation. The air flow rate was held constant at 80 SCFM, and the pressure in the tank was maintained at 10 lb/in^2 gauge. The agitator speed was fixed at 195 rpm.

The secondary vegetative inoculum, 5% by volume, was inoculated into a fermentation medium containing (per liter): Cerelose, 2 g; yeast, 2.5 g; Kaysoy, 20 g; corn starch, 10 g; NH_4Cl, 5 g; and lard oil 10 ml. Sufficient sodium hydroxyide solution was added to adjust the pre-sterilization pH to 7.2.

The fermentations were continued for 92 h. Ucon (Union Carbide, New York, N.Y.) was added automatically as a defoaming agent; about 5,800 ml was the average consumption.

Isolation of crystalline U-42,126 and U-43,795. Four 5,000-liter fermentors initially charged with 4,000 liters as described were harvested after 92 h and



U-42,126 R=H

U-43,795 R=OH

FIG. 1. *The structures of U-42,126 and U-43,795.*

processed by the following scheme. Approximately 3,800 liters of fermentation beer at harvest pH of 7.8 was mixed with 4% Celatom FW-40 and filtered through 42 chambers of a 30-inch (ca. 76 cm) filter press.

The clarified broth containing 43.6 kg of solids estimated to be 0.26% U-42,126 by bioassay was passed through 283 liters of Dowex 50 × 16 (H cycle) in an agitated bed column (61 cm/diameter) at 12.3 liters/min. The pH of the effluent was monitored, and the effluent was successively passed through two trail columns to absorb small amounts of U-42,126 and U-43,795 which were not sorbed by the lead column. When the lead column was considered loaded, it was removed from the train and the small amount of clarified broth remaining was fed directly over the other two trail columns. Each lead column was washed with water and eluted with 1,500 liters of 2.5 N aqueous ammonia at 3 liters/min. After the four runs were finished, the two partially loaded trail columns were eluted in the same way. The elute from each lead column was divided into eight fractions which were evaluated by bioassay. The first 1,000 liters, containing 2,750 g of solids estimated to be 3.1% U-42,126, was pooled and concentrated to 80 liters under reduced pressure to remove ammonia.

The concentrate was percolated through 29 liters of Amberlite IR-45 (OH cycle) in a 15.2-cm column at 1 liter/min. The column was then washed with water, 50% methanol-water, and 90% methanol-water. The column was finally eluted with methanol-water-glacial acetic acid (90:10:3) while the pH of the effluent was monitored. The eluate from the column was split into three fractions: forerun, active, and trail, based on the pH profile. The active fraction was collected from the time the pH started dropping from 7 until it leveled off at 3.7. The three fractions were evaluated by bioassay. The active fraction, 77 liters containing 219 g estimated to be 22% U-42,126, was evaporated to dryness to remove the acetic acid. The forerun, 58 liters containing 55 g of 4.7% U-42,126, and trail, 75 liters containing 94 g of 10% U-42,126, were concentrated for recycling on Amberlite IR 45 (OH form).

The residue from the active fraction was redissolved in 2 liters of water and evaporated onto 1,100 g of silica gel (Silica Gel 60, E. Merck, Darmstadt, Germany). The loaded silica gel was then poured on top of a column (22.5 cm in diameter) containing 25 kg of Silica Gel 60. The column was eluted with methyl ethyl ketone-acetone-water (65:20:15), and the effluent was monitored by thin-layer chromatography on silica gel plates with the same solvent system. U-42,126 and U-43,795 zones were detected by spraying the plates with an acetone solution containing 1% ninhydrin (Eastman Kodak Co.) and 1% pyridine. The initial 102 liters was discarded; the next 43 liters, containing U-43,795, was concentrated to 3 liters of aqueous concentrate. This was clarified by filtration, concentrated to a heavy suspension, diluted with methanol, and chilled to yield 22.8 g of pure crystalline U-43,795. The following 37 liters, containing both U-43,795 and U-42,126, was concentrated for recycling on Amberlite IR 45. The final 172 liters, containing U-42,126, was concentrated to 4 liters of aqueous concentrate and clarified by filtration. Further concentration to a heavy suspension, dilution with methanol, and chilling afforded 34.4 g of pure crystalline U-42,126.

These columns were repeated for each of the four 4,000-liter fermentations and for the partially loaded trail columns of Dowex 50 × 16. In addition, a pool of material for a salvage run was collected from IR 45 forerun and trail fractions, impure silica gel fractions, and crystallization mother liquors. This material was recycled to the IR 45 column and processed as described above.

Microbiological assay. A substantially more sensitive microbiological assay over that previously reported (5) was developed. The assay medium was the previously described (2) completely synthetic agar. It was inoculated with spores of *Bacillus subtilis* UC-902 (1 ml/liter; viable count 7×10^{11} cells/ml) and the assay plates were prepared in the conventional way. The crystalline U-42,126 was diluted in water to reach concentrations suitable for standard curve: 0.1, 0.077, 0.059, 0.045, and 0.035 μg/ml. Such solutions were kept at 4 C for several weeks without any detectable loss of potency. Both standard solutions and unknown samples were applied to 1/2-inch (ca. 1.3 cm) paper disks (0.08 ml/disk), usually in four replications. The assay plates were incubated at 40 C for 18 to 20 h, and the zones of inhibition were recorded. A comparable technique is used for assay of U-43,795. However, because of its lower in vitro activity, concentrations of of 16, 8, 4, and 2 μg/ml are suitable for the construction of standard curve.

Reversal (antagonism) studies. During the early studies done with whole fermentation liquors or partially purified preparations it was possible to prevent the inhibition by U-42,126 of the susceptible microorganism by incorporating histidine into the cultivation media. When preparations of the drugs became available, a more detailed study was conducted. Assay plates inoculated with *B. subtilis* UC-902 were prepared as described above. For some plates the synthetic agar was further supplemented with different concentrations of several potential reversing (antagonizing) agents. The drug was applied at six increasing concentrations on 1/2-inch paper disks. Then the assay plates were incubated for 18 h at

40 C, and the resulting zones of inhibition were recorded. An identical experiment was carried out to study the reversal pattern of U-43,795.

RESULTS AND DISCUSSION

Fermentations and isolation. Table 1 presents the results of a typical fermentation run in 500-ml flasks done under the original fermentation conditions (3) and one carried out with the improved methods.

It was our experience that by using the new fermentation conditions the titers of the drug were increased 30- to 40-fold.

The titers of U-42,126 reached after 92 h of incubation in 5,000-liter tanks were (μg/ml): tank number 1, 33; tank 2, 43; tank 3, 49; and tank 4, 35.

The titers of U-43,795 could not be estimated by *B. subtilis* assay since its activity was less than 0.1% that of U-42,126; however, the drugs were apparently produced in roughly equivalent amounts based on isolated yields.

Successive processing of 16,000 liters of clarified fermentation broth with Dowex 50 (H form) and then Amberlite IR 45 (OH form) afforded a mixture of the two agents. A relatively efficient resolution on silica gel afforded readily crystallized fractions of each component yielding, after recrystallization, 169 g of pure U-42,126 and 167 g of pure U-43,795.

Microbiological assay. A typical dose-response line of U-42,126 on *B. subtilis* UC-902 is presented in Fig. 2. The assay can detect concentrations of 0.03 μg of U-42,126 per ml. The lowest detectable concentration of U-43,795 was 2.0 μg/ml. The slope of dose-response curve was about 6 mm for a twofold concentration increase, and the angle of the slope was comparable to that of U-42,126.

TABLE 1. *Production of U-42, 126 by S. sviceus under two fermentation methods in 500-ml flasks*

Time (h)	Original fermentation conditions[a]		Improved fermentation method[b]	
	Drug titer (μg/ml)	pH	Drug titer (μg/ml)	pH
48	1.4	8.1	5.2	6.0
72	1.1	8.2	27.5	7.7
96	1.0	8.0	34.0	7.8
120	1.0	8.0	41.0	7.8

[a] The original production medium contained (per liter of tap water): starch, 10 g; mannitol, 10 g; Phytone, 10 g (BBL, Cockeysville, Md.); Kaysoy, 10 g, 200 C (Archer Daniels, Midland Co., Decatur, Ill.); $CaCO_3$, 5 g; and NaCl, 2 g. The fermentation was carried out in 500-ml stippled flasks (with 100 ml of media) on a rotary shaker (250 rpm) at 32 C.

[b] The improved production medium was that used in the 5,000-liter tanks.

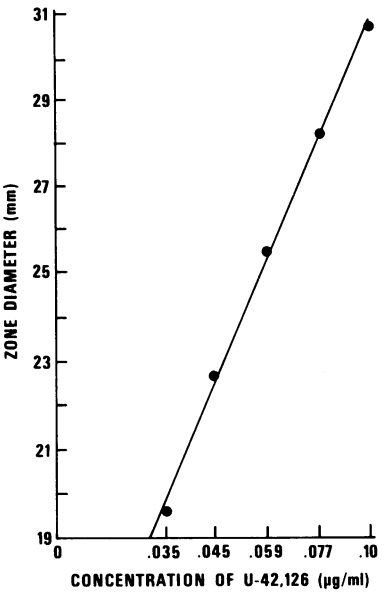


FIG. 2. *Microbiological assay with B. subtilis (UC-902) for U-42,126 in water.*

Incubation of the assay plates at 39 to 40 C is superior to the commonly used 37 C. The sensitivity of the assay can be further increased by ~70% by cooling the spotted assay plates for 2 to 3 h at 4 C prior to the incubation.

Reversal (antagonism) studies. The in vitro antimicrobial effect of U-42,126 was antagonized quite strongly by histidine (Table 2). In other experiments we established that of all the common purines and pyrimidines tested, only deoxyinosine demonstrates slight antagonism. None of the vitamins or any other amino acids (except histidine) prevented the in vitro effect of U-42,126. The antagonism by histidine was rather unusual, though. It was demonstrable at concentration as low as 0.015 μg/ml and kept increasing until the level of 1.0 μg/ml was reached. However, any further increase in its concentrations had no additional reversing effect. The zones of inhibition were about the same on plates containing 1.0, 12.5, or 100 μg of histidine per ml as supplement in the cultivation media. This is a rather unusual situation and is in complete disagreement with our past experience with such antimetabolites as psicofuranine (1) or 5-azaycytidine (3). One of the possible explanations of this unusual observation could be the presence of small amounts of another material that is in vitro active against the test microorganism but that is not antagonized by histidine. However, elemental analysis

TABLE 2. *Inhibition by U-42, 126 of B. subtilis UC-902 cultivated in synthetic agar supplemented with histidine*

Medium supplement (μg/ml)	Concn of U-42, 126 (μg/ml)					
	0.5	0.25	0.125	0.062	0.031	0.015
Control (no supplements)	41[a]	36	31	25	18	0
Histidine (0.015)	40	34	27	19	0	0
Histidine (0.03)	39	33	26	t	0	0
Histidine (0.06)	39	32	24	t	0	0
Histidine (0.12)	37	31	21	0	0	0
Histidine (0.25)	35	27	15	0	0	0
Histidine (0.5)	25	t	0	0	0	0
Histidine (1.0)	20	t	0	0	0	0
Histidine (12.5)	22	t	0	0	0	0
Histidine (100)	20	t	0	0	0	0

[a] The numbers in the body of the table are dimaeters of the zones of inhibition (expressed in millimeters) around 12.7-mm paper disks. t, Trace.

TABLE 3. *Inhibition by U-43, 795 of B. subtilis cultivated in synthetic agar supplemented with histidine*

Medium supplement (μg/ml)	Concn of U-43, 745 (μg/ml)			
	20	10	5	2.5
Control (no supplements)	48[a]	32	26	19
Histidine (0.62)	37	31	21	0
Histidine (1.25)	36	29	18	0
Histidine (2.5)	34	27	t	0
Histidine (5.0)	30 (h)	28 (h)	0	0
Histidine (10.0)	21 (h)	t	0	0
Histidine (20.0)	21 (h)	t	0	0

[a] The numbers in the body of the table are diameters of the zones of inhibition (expressed in milliliters) around 12.7-mm paper disks. h, Hazy; t, trace.

(C, H, N, Cl, and equivalent weight), spectral analysis (infrared, ultraviolet, nuclear magnetic resonance, and circular dichroism), and chromatographic analysis (silica gel and paper) indicated that the sample utilized for histidine reversal studies was homogenous and consistent with the assigned structure. The reversal pattern of U-43,795 was comparable to that of U-42,126 (Table 3).

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