A32390A, A NEW BIOLOGICALLY ACTIVE METABOLITE

I. DISCOVERY AND FERMENTATION STUDIES

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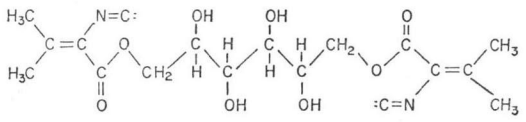
A32390A is an isonitrile-containing derivative of diacyl D-mannitol. The compound is produced in fermentation as the major component of a metabolic complex known as A32390. A32390A inhibits dopamine-β-hydroxylase, reduces heart and adrenal norephinephrine levels, lowers blood pressure in hypertensive rats, and possesses antibiotic activity *vs.* Gram-positive bacteria and fungi, including *Candida albicans*. A32390 is produced in submerged culture by a mold, a species of *Pyrenochaeta*, NRRL-5786. Glucose and sucrose are among the best carbon sources for the biosynthesis of A32390. Mannitol, although a substituent of the A32390A molecule, supports little or no biosynthesis of the compound when employed as the major carbon source for the fermentation. The addition of crotonic acid derivatives, ethanol, or L-histidine to the fermentation medium enhances the level of A32390 produced.

A32390A is an isonitrile-containing derivative of a diacyl hexitol with the absolute configuration of D-mannitol; 1,6-di-O-(2-isocyano-3-methylcrotonyl)-D-mannitol (Fig. 1)[1]. The compound is produced by a novel strain of fungus isolated from a subtropical soil sample and classified as a species of the genus *Pyrenochaeta*, a member of the order *Sphaeropsidales* in the Deuteromycetes.

A32390A has a molecular weight of 396, is insoluble in water, and unstable in alcohols[1]. It is the major component of an antibiotic complex that contains small amounts of at least three additional factors. The complex, known as A32390, was discovered during a screening program designed to detect the production of pharmacologically-active metabolites other than antibiotics. Antibiotic activity was subsequently observed with concentrated solvent extracts of the broth. Crystalline A32390A was eventually shown to possess both dopamine-β-hydroxylase (DBH) and antimicrobial activities[1].

A32390A inhibits DBH non-competitively *in vitro*. Intraperitoneal or subcutaneous administration to rats produces a dose-dependent reduction in the norepinephrine levels of heart and adrenal tissue. Lowering of blood pressure has been shown in hypertensive rats. Oral administration of A32390A was not effective[2].

Fig. 1. Structure of A32390A.



1,6-Di-O-(2-isocyano-3-methylcrotonyl)-D-mannitol

Fungistatic *in vitro* activity was demonstrated against several test organisms, including *Candida albicans*. *In vivo* activity against *C. albicans* was also observed after intraperitoneal or subcutaneous administration of A32390A[3].

Naturally-occurring isonitrile compounds are relatively rare. Those described in the literature include only a pair of sesquiterpenoids from a marine sponge[4]; antibiotic B371, an indoleacrylo-

isonitrile produced by *Pseudomonas*[5]; an isocyano epoxide isolated from the culture filtrate of *Trichoderma*[6]; and the xanthocillins, a family of antibiotics produced by *Penicillium notatum*[7,8]. The carbon skeleton and nitrogen atoms of xanthocillin are known to be derived from tyrosine but the origin of the isonitrile carbon has not been elucidated[9,10].

This communication reports discovery of the A32390 complex and the results of initial fermentation studies.

Quantitation of Activity in Fermentation Samples

A32390 was initially detected and monitored by an enzymatic technique that employed radioisotopes to measure the inhibition of dihydroxyphenylalanine (dopa) formation by tyrosine hydroxylase[11]. The discovery of antibiotic activity in concentrated broth extracts (Table 1) led to the development of a conventional disc-plate agar diffusion assay employing *Micrococcus luteus* (PC 1001, FDA) as the test organism. Test samples were diluted in 0.1 M phosphate buffer at pH 6.0. The assay medium contained 0.15% beef extract, 0.3% yeast extract, 0.6% peptone, and 1% Ionagar No. 2 (Colab). Eight ml of inoculated medium at pH 6.0 was dispensed into 85-mm circular plates. Test plates were incubated overnight at 37°C. Activity was measured against a lyophilized fermentation broth that served as a reference standard until a crystalline preparation of A32390A became available. Fermentation titers are expressed in terms of the crystalline A32390A standard.

Table 1. *In vitro* activity of initial A32390 samples

Test	Sample	
	Filtered broth	Concentrated butanol extract of filtrate
Enzymatic (dopamine-β-hydroxylase)	96[a]	98[a]
Bacterial (*Micrococcus luteus*)	Not active	21[b]
Fungal (*Neurospora crassa*)	Not active	18[b]

[a] Percent inhibition. [b] Zone diameter in mm.

Stability in Fermentation Broths

Broth filtrates contained 85~90% of the A32390 activity produced by the fermentation. Preliminary indications of declining activity in older samples led to the evaluation of several parameters known to affect the stability of other antibiotics. The effect of pH is shown in Table 2. Stability was best at neutrality, the normal pH at harvest, but declined sharply outside the range of pH 6~8. Samples held at pH 7.0 and 25°C for 72 hours prior to reassay versus a freshly prepared standard curve lost 35~40% of their initial activity. The activity of similar samples held under refrigeration for the same period, however, declined only 10%.

Table 2. Effect of pH on stability of A32390 in fermentation broth filtrates

pH of sample	Antibiotic titer (mcg/ml) after holding at 6°C	
	6 hours	24 hours
4	8	<5
5	13	<5
6	19	16
7	21	18
8	20	16
9	7	<5
10	<5	

Zero-time titer at pH 7.0 was 21 mcg/ml. Titers were corrected for volume increase due to acid and base addition.

Chromatography

Fermentation samples were monitored qualitatively in a variety of chromatography systems. The system ultimately adopted for routine use employed thin-layer silica gel G plates developed in chloroform - methanol (9: 1). Developed chromatograms were bioautographed on agar plates seeded with *M. luteus*. The Rf values of the various factors of the A32390 complex in this system are shown in Table 3.

Table 3. Rf Values of individual A32390 factors.

Factor	Rf
A	0.32
B	0.23
C	0.15
D	0.05

Silica gel G plates developed in $CHCl_3$ - MeOH (9: 1) and bioautographed on *M. luteus*.

Culture Characteristics, Preservation and Propagation

Most species of the Order *Sphaeropsidales* grow either parasitically or saprophytically on plant materials. The A32390-producing *Pyrenochaeta* culture, NRRL-5786, grew well on a variety of agar media, producing colonies of 30~40 mm diameter in 8 days at 25°C. Equivalent amounts of growth were obtained over a temperature range of 18~30°C. Decreased growth was observed as the temperature was elevated above 30°C. The culture did not grow at 40°C. Both the obverse and reverse surfaces of colonies were brownish-black. Aerial hyphae, profuse on some media but sparse on others, were usually whitish-gray. Pycnidia, formed most abundantly on BENNETT's or potato-dextrose agars, were superficial, cespitose, membranous to subcarbonaceous, and setose. Normally black and subglobose with an open and slightly rostrate ostiole, they averaged 170 microns in width and 195 microns in length. A mucoid mass of unicellular spores was discharged through the ostiole. The hyaline, cylindrical spores possessed rounded ends, averaged 2.8 × 8.4 microns, and were elaborated from conidiogenous cells that projected from the pycnidial wall. Tapered from base to apex, the conidiogenous cells averaged 22 microns in length.

Stock cultures were initially propagated by serial transfer on a medium containing 2% glucose, 0.5% Bacto-Peptone (Difco), 0.05% KH_2PO_4, 0.002% $MgSO_4 \cdot 7H_2O$, and 2% washed agar in deionized water. The post-autoclaving pH was 5.5. After an incubation period of 8~10 days at 25°C, the culture was immediately suspended in calf serum and lyophilized. Mature cultures on agar media exhibited a marked reduction in viability after refrigeration for periods as short as 14 days. Although viability and A32390-producing capability were retained through the lyophilization process, later investigation determined that submerged cultures grown as fermentor inoculum and preserved in the vapor phase of liquid nitrogen (LN)[12] grew more luxuriously upon transfer to new media and consistently produced higher titers of A32390.

Fermentor inoculum was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of a medium composed of 2.5% sucrose, 3.6% molasses, 0.6% corn steep liquor, 1% malt extract, 1% tryptic digest of casein, 0.2% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.02% KCl in deionized water. These flasks were inoculated either from agar slant cultures or, preferably, from liquid nitrogen tubes, and incubated 48 hours at 25°C on a shaker rotating at 250 rpm in a 5-cm diameter circle. The resulting culture was used to provide a 1% (v/v) level of inoculum to fermentors.

Fermentation Studies

Fermentation studies were conducted in wide-mouth 250-ml Erlenmeyer flasks that contained 50 ml of media. Incubation conditions were the same as those described for fermentor inoculum. All media were prepared with deionized water. The initial fermentation conditions and several later modifications are shown in Table 4. Unless otherwise indicated, terminal pH values were 6.8~7.2 and growth, measured as packed solids after centrifugation in conical tubes for 15 minutes at 1,000×g, was 25~35%. The first increase in the level of A32390 was achieved through the use of a new strain, WB8, that was maintained by LN storage (Table 4).

Table 4. Fermentation conditions employed for production of A32390A

	Medium				
	A	A	B	C	D
Glucose			1.5	1.5	1.5
Sucrose	3.0[a]	3.0	3.0	3.0	3.0
Amber BYF 300					1.0
Cottonseed meal	0.5	0.5	0.5	0.5	
$NaNO_3$	0.05	0.05	0.1	0.1	
K_2HPO_4	0.1	0.1	0.2	0.2	
KCl	0.07	0.07	0.07	0.07	
L-Histidine				0.01 M	
$MgSO_4 \cdot 7H_2O$	0.07	0.07	0.07	0.07	
$FeSO_4 \cdot 7H_2O$	0.0014	0.0014	0.0014	0.0014	
Strain:	Wild type[b]	WB8[c]	WB8	WB8	WB8
Fermentation time (days):	3	3	3	4	4
Antibiotic titers (mcg/ml):	±5	33	51	157	86

[a] Expressed as percent. [b] Preserved by lyophilization. [c] Maintained by LN storage.

Effect of Various Carbon Sources A number of carbohydrates, sugar alcohols, and oils were tested individually in a basal medium from which the standard carbohydrate, sucrose, had been omitted (Table 5). The rate and level of growth produced by all of the substrates was very similar except that 25~30% less growth was achieved with the sugar alcohols, sorbitol and mannitol. Substrate levels of 2~8% supported comparable amounts of growth. Sorbose, the sugar alcohols, and the oils, however, supported little or no A32390 biosynthesis. Glucose and mannose produced the highest titers of A32390. Yields with fructose and maltose were slightly lower. Although inversion of sucrose yields equimolar quantities of glucose and fructose, each of the hexoses was superior to the disaccharide. Higher levels of the ketose repressed A32390 biosynthesis sharply, however, while the aldose did not. Further examination of various levels and combinations of the most promising sugars determined that the optimum level was 4.5% and maximum titers of A32390 were produced by a glucose-sucrose combination. Medium B in Table 4 reflects these findings. Because A32390A contains a D-mannitol moiety, the effect of mannitol on A32390 yields was of particular interest. The fact that little or no A32390 is produced when mannitol serves as the carbon source suggests a strong feedback effect on A32390A biosynthesis by mannitol. Since glucose is one of the best carbohydrates for the production of A32390A, the D-mannitol moiety is presumably

derived from glucose. D-Mannitol production from D-glucose in 75% yield has been reported for *Aspergillus candidus*[13].

Crotonic Acid Derivatives Preliminary structure determinations suggested the isocyano substituents (–N=C) of A32390A were cyano groups (–C=N). Biosynthesis of cyanide by microbes does occur, having been reported in *Pseudomonas, Chromobacterium, Bacillus* and fungi[14]. Further, *Nocardia rhodochrous* utilizes several nitriles to supply carbon and nitrogen for growth[15]. Several derivatives of 2-cyano-3-methyl crotonate were therefore examined for possible enhancement of A32390A production (Table 6). All four of the compounds tested stimulated antibiotic titers, with the ethyl ester and the methoxyethyl ester being most stimulatory. Optimum levels were 0.05~0.1%. The mechanism of this stimulation in light of the true structure of A32390A is not known. Interconversion of cyano to isocyano substituents or the biosynthesis of new cyano compounds that were not recognized chromatographically may have occurred.

Lower Alcohols Concern regarding stability of the crotonic acid derivatives during autoclaving led to efforts to filter sterilize these compounds. Because of their low aqueous solubility, they were dissolved in 95% ethanol prior to filtration through Millipore Fluoropore filters (0.2 μm) and an ethanol control was employed. The ethanol controls (1.4%) produced 65% more activity than the standard controls (Table 7). Fermentations containing ethanol were also characterized by more homogeneous growth, reddish-brown pigmentation, and a "sweet" odor. Methanol produced little if any stimulation but 2-propanol, acetone, and ethyl acetate produced stimulation of approximately the same magnitude as ethanol. Although the mechanism of this stimulation on the A32390 fermentation is not known, the use of alcohols as a carbon source is widely recognized. In addition,

Table 5. Effect of various carbon sources on the growth and biosynthesis of A32390 by *Pyrenochaeta*[a]

Carbon source[b]	Growth (% Solids)	Antibiotic titer (mcg/ml)
—[c]	7	<5
Sucrose	28	31
Glucose	26	44
Fructose	30	38
Sorbose	26	<5
Sorbitol	18	<5
Mannose	28	41
Mannitol	21	<5
Maltose	29	34
Dextrin	40	22
Cottonseed oil	31	<5
Corn oil	30	<5
Peanut oil	34	<5
Soybean oil	29	<5
Lard oil	38	<5

[a] Medium A minus sucrose.
[b] Initial concentration of carbohydrates was 3%, oils 4%.
[c] Terminal pH was 8.6.

Table 6. Effect of 2-cyano-3-methyl crotonate derivatives on A32390 production

Addition[a]	A32390 titer (mcg/ml)
None[b]	33
Acid	48
Amide	58
Ethyl ester	77
Methoxy-ethyl ester	69

[a] 0.1% level. [b] Control was Medium A, Table 4

Table 7. Effect of lower alcohols, acetone, and ethyl acetate on production of A32390

Addition[a]	A32390 titer (mcg/ml)
None[b]	33
Ethanol	55
Methanol	37
2-Propanol	58
Acetone	51
Ethyl acetate	62

[a] 1.4% level. Filter sterilized.
[b] Medium A, Table 4.

alcohols occasionally appear to exert other, sometimes unexplained, effects on microbial cultures. For example, a 3% concentration of methanol increases citric acid production by *Aspergillus niger* up to 50~60% in surface or solid state, but not submerged, culture[16]. A 0.5~2% level of ethanol and other alcohols also increases L-proline production by *Corynebacterium glutamicum*, possibly through growth regulation and/or effects on cell permeability[17].

Amino Acids Enrichment of the fermentation medium with common amino acids was examined to determine whether a specific amino acid stimulated the biosynthesis of A32390A. A beneficial effect on antibiotic titers was observed with the racemic form of four amino acids; phenylalanine, tyrosine, valine, and histidine (Table 8). Because histidine was most stimulatory, the D and L-isomers were tested individually. The stimulatory effect was due specifically to the L-isomer. Omission of cottonseed meal from Medium B (Table 4) reduced antibiotic titers to <5 mcg/ml. Enrichment of this medium with phenylalanine, tyrosine, valine, or L-histidine restored antibiotic titers to a level of 15~20 mcg/ml. Under these conditions, however, histidine was not superior to the other amino acids. The stimulation by histidine observed in Medium C therefore presumably resulted from a regulatory effect under those conditions that may not be duplicated in other media.

Proteins and Peptones. Cottonseed meal, at a level of 0.5%, was a standard component of the fermentation medium. Omission of this substrate reduced growth from 28% to 5% and concurrently reduced A32390A titers from 33 to <5 mcg/ml (Table 9). Substitution of either soybean meal, fish meal, peanut meal or Amber BYF for cottonseed meal increased antibiotic yields by 55~79%. Further work with these substrates determined that higher levels of only one, Amber BYF, provided consistent enhancement of A32390A titers. These data led to the development of Medium D (Table 4), which, al-

Table 8. Effect of amino acid enrichment on production of A32390

Amino acid addition	A32390 titer (mcg/ml)
None[a]	51
DL-Phenylalanine[b]	76
DL-Tyrosine	64
DL-Valine	67
DL-Histidine	104
D-Histidine	59
L-Histidine	157

[a] Medium B, Table 4.
[b] Level of addition was 1×10^{-2} M.

Table 9. Effect of various proteinaceous substrates on biosynthesis of A32390A

Substrate	Substrate level (%)	Growth (% Solids)	A32390A titer (mcg/ml)
None		5	<5
Cottonseed meal[a]	0.5	28	33
Meat peptone	0.5	8	23
Enzyme-hydrolyzed casein	0.5	22	31
Soybean meal	0.5	30	51
	1	26	50
Fish meal	0.5	24	54
	1	28	58
Peanut meal	0.5	34	59
	1	41	51
Amber BYF[b]	0.5	18	52
	1	29	89
	1.5	32	83

[a] Medium A, Table 4. [b] Amber Laboratories.

though containing only 3 components, produced higher titers of A32390A than Medium B.

Fermentation Time-course. A typical time-course of the fermentation in a 165-liter fermentor using Medium C (Table 4) is shown in Table 10. The pH declined slowly from 6.9 to 5.9 during the trophophase and increased gradually during the idiophase. Little biosynthesis of A32390 occurred until completion of the trophophase on about the third day of the fermentation. Antibiotic titers then increased rapidly for approximately 24 hours, reaching peak values late on the fourth day. Although other fermentation parameters remained relatively constant during an additional 24-hour period, A32390 titers decreased sharply after maximum levels were attained, frequently declining more than 50% by the fifth day.

Table 10. Typical time-course of A32390 fermentation in 165-liter fermentor[a]

Fermentation age (hours)	pH	Biomass (% Solids)	Antibiotic titer (mcg/ml)
0	6.9	<1	
17	6.7	4	
41	6.3	9	<5
70	5.9	24	14
89	6.2	28	124
97	6.5	27	161
113	6.7	29	76

[a] Medium C, Table 4.

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