# A32390A, A NEW BIOLOGICALLY ACTIVE METABOLITE

## II. ISOLATION AND STRUCTURE

Gary G. Marconi,* Bryan B. Molloy, R. Nagarajan, James W. Martin, Jack B. Deeter and John L. Occolowitz

The Lilly Research Laboratories
Eli Lilly and Company
Indianapolis, Indiana 46206, U.S.A.

(Received for publication October 14, 1977)

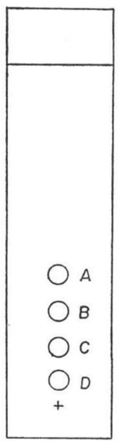
An inhibitor of dopamine-$\beta$-hydroxylase, designated A32390A, was isolated from the culture broth of a *Pyrenochaeta* species. The inhibitor showed antimicrobial activity against fungi and gram-positive bacteria. Spectroscopic analysis and chemical degradation studies indicated that the structure was 1,6-di-O-(2-isocyano-3-methylcrotonyl)-D-mannitol.

In the course of screening for microorganisms that produce dopamine-$\beta$-hydroxylase inhibitors, a culture was found that produced several compounds that inhibited the enzyme. The producing organism (NRRL 5786) was classified as a strain of the genus *Pyrenochaeta* in the order Sphaeropsidales. Fermentation conditions for the production of the inhibitor are described in the preceding paper[1]. A thin-layer chromatogram of filtered broth is shown in Fig. 1. Chromatograms were developed on pre-coated Merck silica gel F-254 plates using chloroform - methanol (9: 1) as the mobile phase in a saturated chamber. The components were visualized using iodine vapor, short wavelength UV light, or by bioautography on *Micrococcus luteus*. Four compounds, designated A32390A, B, C and D, were detected by bioautography. The total amount of these compounds in the broth was estimated by microbiological assay to be 60 $\mu$g/ml.

### Isolation of A32390A

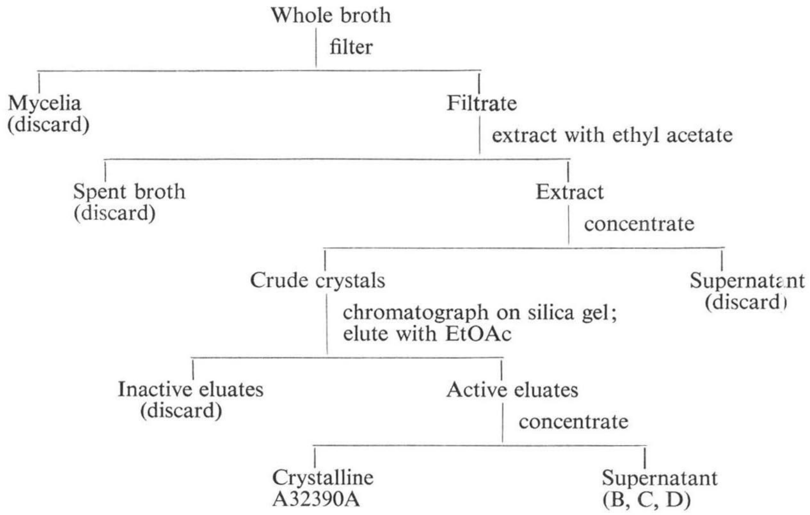
The procedure for the isolation of A32390A is shown schematically in Fig. 2. Hyflo Supercel was added to whole broth which was then filtered to remove the mycelia. The broth filtrate was extracted twice with ethyl acetate at harvest pH and the combined extracts were concentrated to approximately 1% of the original broth volume. When the concentrates were cooled, a precipitate formed that was 75% by weight A32390A. The precipitate was collected and chromatographed on a Grade 62 silica gel column using ethyl acetate as the eluant. Fractions were monitored microbiologically using *M. luteus* as the assay organism. The active fractions were combined, concentrated, and cooled

Fig. 1. Thin-layer chromatogram of the A32390 complex.




Solvent:
Chloroform - methanol (9: 1)
Sorbent:
Merck Silica Gel F-254
Organism:
*Micrococcus luteus*

Fig. 2. Isolation procedure for A32390A.




to obtain crystals of A32390A.

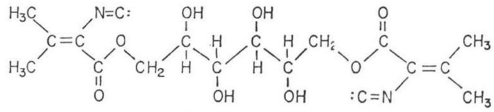
## Structure of A32390A

A32390A was assigned the structure 1,6-di-O-(2-isocyano-3-methylcrotonyl)-D-mannitol (Fig. 3) based on the following data.

The infrared spectrum of A32390A (Fig. 4) contains a sharp band at 2150 $cm^{-1}$ due to an isonitrile, a functional group rarely found in natural products[2,3,4]. The hydroxyl groups display an intense band at 3450 $cm^{-1}$. Bands at 1740 $cm^{-1}$ and 1630 $cm^{-1}$ are due to the ester carbonyl and C-C double bond, respectively.

The 100 MHz $^1H$ NMR spectrum of A32390A in DMSO-$d_6$ is shown in Fig. 5. The molecule is symmetric about the $C_3$-$C_4$ bond (Fig. 3) and as a result the chemical shift of a proton in one-half of the molecule is identical to the chemical shift of the corresponding proton in the other half. Thus, each signal in the spectrum represents two protons. The pair of three proton singlets at 2.1 and 2.3 ppm is assigned to the methyl groups of the crotonic acid moiety and the two proton

Fig. 3. Structure of A32390A.



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

Fig. 4. Infrared spectrum of A32390A (Mull).

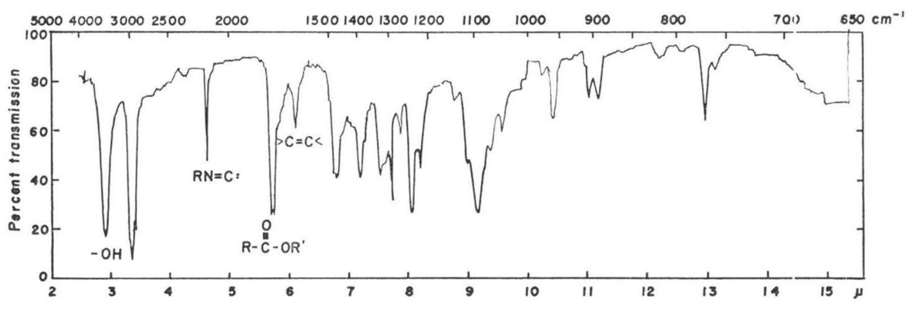
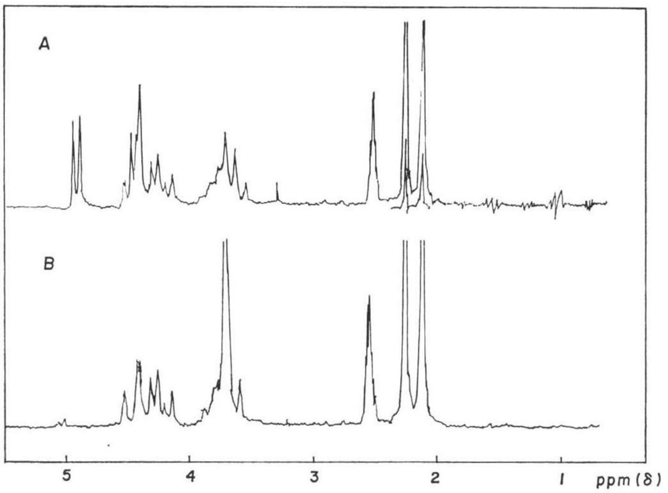


Fig. 5. 100 MHz $^1H$ NMR spectrum of A32390A in A) DMSO-$d_6$, B) DMSO-$d_6$/$D_2O$.




multiplet at 3.7 ppm arises from the methine protons of the hexitol. The two doublets centered at 4.4 and 4.0 ppm disappear on addition of $D_2O$ and are assigned to the hydroxyl protons. After the $D_2O$ exchange, each methylene proton is visible as a pair of doublets, one at 4.2 ppm and one at 4.45 ppm. These assignments were confirmed by decoupling experiments.

Hydrolysis with methanolic potassium carbonate cleaves A32390 into two fragments. One fragment was isolated and identified as N-formyldehydrovaline. The other fragment, a hexitol, was acetylated with acetic anhydride in pyridine. A hexaacetate was isolated and compared with an authentic sample of D-mannitol hexaacetate. The melting points and mixed melting point (126~127°C), optical rotation ($[\alpha]_D^{27}$ +25.2°), and X-ray diffraction patterns of the two samples were identical.

The tetraacetyl ester of A32390A was prepared using acetic anhydride in pyridine. The corresponding propionyl and butyryl esters were prepared in a similar manner using the appropriate anhydride.

A32390A was transesterified by simply dissolving the compound in one of the following alcohols: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, or *t*-butanol. The products of the transesterification reaction were D-mannitol and the corresponding isocyanomethyl crotonyl ester. Reduction of A32390A tetraacetate with hydrogen and palladium on carbon produced 1,6-di-O-(N-methylvalyl)-D-mannitol tetraacetate.

## Biological Activity

A32390A has *in vivo* activity against *Candida albicans* in addition to the *in vitro* antimicrobial activity shown in Table 1. Details of these experiments are published elsewhere[5].

A32390A was found to be an inhibitor of dopamine-$\beta$-hydroxylase with an $I_{50}$ of 1.7 $\mu$g/ml (fusaric acid[6] has an $I_{50}$ of 0.4 $\mu$g/ml in our assay). The compound lowers catecholamine levels in the heart and adrenals of rats with subsequent lowering of blood pressure when administered parenterally (Tables 2 and 3)[7].

The acute toxicity of A32390A was low with an $LD_{50}$ of greater than 1,000 mg/kg (i.p., mice).

Table 1. *In vitro* antimicrobial activity of A32390A and its tetraacyl esters at 1 mg/ml (6 mm discs)

| Organism | Zone Diameter (mm) | | | |
|---|---|---|---|---|
| | Factor A | Tetraacetate | Tetrapropionate | Tetrabutyrate |
| *Staphylococcus aureus* | 30 | 20 | 12 | trace |
| *Bacillus subtilis* | 30 | 20 | 11 | — |
| *Micrococcus luteus* | 28 | 24 | 12 | 9 |
| *Mycobacterium avium* | 26 | 10 | — | — |
| *Saccharomyces pastorianus* | 24 | 24 | 22 | 10 |
| *Neurospora crassa* | 20 | 18 | 16 | 9 |
| *Candida tropicalis* | 19 | 15 | 14 | — |
| *Fusarium moniliforme* | — | 28 | 17 | — |
| *Trichophyton mentagrophytes* | — | 22 | 11 | — |
| *Escherichia coli* | 30 | 27 | 19 | — |
| *Pseudomonas solanacearum* | — | — | — | — |

Table 2. Heart noradrenaline level in male Harlan rats after single intraperitoneal injections of A32390A[6,7)]

| Dose A32390A (mg/kg) | Noradrenaline levels | |
|---|---|---|
| | mcg/g tissue | % |
| 0 (Control) | 0.87±0.03 | 100 |
| 3 | 0.73±0.04 | 84* |
| 10 | 0.80±0.07 | 92 |
| 32 | 0.62±0.04 | 71* |
| 100 | 0.33±0.07 | 38** |

* $P<0.05$. ** $P<0.01$.

Table 3. Blood pressure of DOCA-NaCl hypertensive rats after single injections of A32390A[6,7)]

| Dose A32390A (mg/kg; i.p.) | Control | Blood pressure (mmHg±s.e.m.)* after administration | | | |
|---|---|---|---|---|---|
| | 0 hr. | 3 hrs. | 6 hrs. | 24 hrs. | 48 hrs. |
| 200 | 233±5 | 164±9 | 160±10 | 157±8 | 213±11 |
| 100 | 205±14 | 163±14 | 159±24 | 175±26 | 163±21 |
| 50 | 221±13 | 140±12 | 147±10 | 202±17 | 217±8 |
| 25 | 218±14 | 154±38 | 152±23 | — | — |

* Mean of four animals.

## Experimental

### Isolation of A32390 Factor A

Fermentation broth (100 liters) was filtered using 5% Hyflo Supercel filter aid. The broth filtrate thus obtained was extracted with two 60-liter portions of ethyl acetate. The combined ethyl acetate extracts were evaporated under vacuum to a volume of 1 liter. This solution was cooled (4°C) for 24 hours. The semicrystalline precipitate which formed was separated by filtration, washed with cold ethyl acetate (50 ml) and dried under vacuum to give the A32390 antibiotic complex (5.0 g). This material was slurried in benzene (100 ml) and was applied to a 3.7×90 cm silica gel (Matheson Grade 62) column prepared in benzene. The column was eluted successively with benzene (2 liters), benzene - ethyl acetate (1:1) (3 liters), and ethyl acetate (500 ml). Further elution with ethyl acetate

(10.5 liters) yielded fractions containing A32390A. These fractions were combined and evaporated to dryness under vacuum. The residue thus obtained was dissolved in 50 ml hot acetone. A32390A crystallized upon cooling. The crystals were separated by filtration, washed with cold acetone, and dried under vacuum; 1.5 g of material was obtained. mp 162°C (dec), $[\alpha]_D^{27}$ +18°C (*c* 1, DMF). UV (EtOH): $\lambda_{max}$ 230 nm ($\varepsilon$ 27,900). IR (Nujol): 3450 $cm^{-1}$ (OH), 2150 $cm^{-1}$ (–N≡C), 1740 $cm^{-1}$ (ester), 1630 $cm^{-1}$ (double bond). NMR (DMSO-$d_6$): 2.11 (s), 2.24 (s), 3.61 (dd, J=9, 7 Hz), 3.77 (m, J=9, 6, 5.5, 2 Hz), 4.21 (dd, J=11, 6 Hz), 4.45 (dd, J=11, 2 Hz), 4.42 (exchangeable d, J=7 Hz) and 4.91 (exchangeable d, J=5.5 Hz). Elemental analysis:

| | | |
|---|---|---|
| Calcd for $C_{18}H_{24}N_2O_8$: | (MW 396) | C, 54.6; H, 6.06; N, 7.07; isonitrile, 13.13. |
| Found: | | C, 54.63; H, 6.03; N, 7.38; isonitrile, 12.47[8]. |

Base Hydrolysis of A32390A

To a solution of 75 mg of A32390A in 10 ml of methanol was added 100 mg of potassium carbonate in 5 ml of methanol - water (1: 1). After stirring the solution for 5 hours, the pH was adjusted to 2.5 with 2 N hydrochloric acid and the solution was evaporated to dryness. The residue was suspended in 95% ethanol and centrifuged. The supernatant was evaporated to dryness and yielded 27 mg of N-formyl dehydrovaline. The NMR spectrum (100 MHz, DMSO-$d_6$) showed the C-methyl groups as singlets at 1.73 and 2.00 ppm, the formyl proton at 3.54 ppm (d, J=1.5 Hz), and the broad amide signal at 4.80 ppm. After adding $D_2O$ to the solution, the formyl doublet collapsed to a singlet and the amide proton signal disappeared.

The ethanol-insoluble material was acetylated with 5 ml of pyridine and 5 ml of acetic anhydride overnight. After evaporation of the solution to dryness, the residue was dissolved in chloroform. The organic solution was washed with hydrochloric acid followed by sodium bicarbonate. Finally, it was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was crystallized from ethanol and was shown to be D-mannitol hexaacetate. (Determined by comparison with an authentic sample *via* optical rotation, mixed melting point, and X-ray diffraction powder pattern.)

Acetylation of A32390A

Acetic anhydride (2.5 ml) was added to a solution of 206 mg of A32390A in 5 ml of dry distilled pyridine. The resulting solution was allowed to stand overnight at room temperature and was then concentrated to dryness. The residue was repeatedly redissolved in chloroform and re-evaporated under vacuum until the resulting residue had no detectable pyridine odor. The final residue, dissolved in 2 ml of chloroform, was loaded onto a silica gel column (Grade 62, 1.5×3.0 cm) equilibrated in chloroform. The column was eluted with chloroform and twenty-five 15 ml fractions were collected. Active fractions (7~11) were combined and concentrated under vacuum to a viscous oil that was dissolved in diethyl ether and reconcentrated. The product was dried under vacuum to give 268 mg of A32390A tetraacetate as a viscous colorless liquid. The mass spectrum showed $M^+$ at 564.193 which is consistent with $C_{26}H_{32}N_2O_{12}$ (mw 564.196). The NMR spectrum showed two singlets at 2.08 and 2.10 ppm corresponding to the added acetate methyl groups. The IR spectrum showed an intense band at 1740 $cm^{-1}$ and the absence of absorption at 3500 $cm^{-1}$ (OH). Elemental analysis:

| | | |
|---|---|---|
| Calcd for $C_{26}H_{32}N_2O_{12}$: | (MW 564) | C, 54.96; H, 5.67; N, 4.96; O, 34.04 |
| Found: | | C, 54.19; H, 6.51; N, 4.16; O, 33.99 |

Ethanolysis of A32390A

A solution of 222 mg of A32390A in 50 ml of 95% ethanol was refluxed for 5 hours. The reaction mixture was evaporated to dryness and the residue was redissolved in 10 ml of chloroform and 10 ml of water. The layers were separated and the water solution was found to contain D-mannitol. The chloroform layer yielded an oily residue which was shown to be ethyl 2-isocyano-3-methyl crotonate.

The mass spectrum showed $M^+$ at 153. The NMR spectrum shows a methyl triplet at $\delta$1.33 (J=7 Hz), a methylene quartet at $\delta$4.28 (J=7 Hz), and two 3-proton methyl singlets at $\delta$2.13 and 2.28.

Hydrogenation of A32390A Tetraacetate

Pre-reduced 5% palladium on carbon (25 mg) was added to a solution of 130 mg of A32390A tetraacetate in 10 ml of ethanol. The reduction was carried out in an atmosphere of hydrogen at atmospheric pressure and ambient temperature for 100 hours. The catalyst was then filtered and evaporation of the filtrate gave 130 mg of 1,6-di-O-(N-methylvalyl)-D-mannitol tetraacetate. The mass spectrum showed $M^+$ at 576.290. Calculated for $C_{25}H_{44}N_2O_{12}$: 576.289. In the NMR spectrum the $-CH(\underline{CH_3})_2$ occurs as a doublet at $\delta$ 0.92 with J=6.5 Hz, and the $-NH\underline{CH_3}$ group as a singlet at $\delta$ 2.34.

Acknowledgments

We wish to thank Dr. P. P. K. Ho for dopamine-$\beta$-hydroxylase inhibition assays and Dr. PAUL DEMARCO for discussions of the NMR data.

## References

1) BOECK, L. D.; M. M. HOEHN, T. H. SANDS & R. W. WETZEL: A32390A, a new biologically active metabolite. I. Discovery and fermentation studies. J. Antibiotics 31: 19~26, 1978
2) HAGEDORN, I. & H. TONJES: Structural elucidation of xanthocillin. X. Pharmazie 12: 567~580, 1957
3) NOBUHARA, M.; H. TAZIMA, K. SHUDO, A. ITAI, T. OKAMOTO & Y. IITAKA: A fungal metabolite, novel isocyano epoxide. Chem. Pharm. Bull. 24: 832~834, 1976
4) EVANS, J. R.; E. J. NAPIER & P. YATES: Isolation of a new antibiotic from a species of *Pseudomonas*. J. Antibiotics 29: 850~852, 1976
5) TURNER, J. R.; T. F. BUTLER, R. S. GORDEE & A. L. THAKKAR: A32390A, a new biologically active metabolite. III. *In vitro* and *in vivo* antifungal activity. J. Antibiotics 31: 33~37, 1978
6) HIDAKA, H.; T. NAGATSU, K. TAKEYA, T. TAKEUCHI, H. SUDA, K. KOJIRI, M. MATSUZAKI & H. UMEZAWA: Fusaric acid, a hypotensive agent produced by fungi. J. Antibiotics 22: 228~230, 1969
7) FULLER, R. W.; P. P. K. HO, C. MATSUMOTO & J. A. CLEMENS: New inhibitors of dopamine-$\beta$-hydroxylase. Advances in Enzyme Regulations 15: 267~281, 1977
8) ROLSKI, S. & G. M. MACIAK: Micro- and ultramicrodetermination of the formyl and isonitrile groups in the presence of other acyl groups. Microchem. J. 18: 181~189, 1973