(+)-(S)-Dihydroaeruginoic Acid, an Inhibitor of Septoria tritici and Other Phytopathogenic Fungi and Bacteria, Produced by Pseudomonas fluorescens

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J. Nat. Prod., **1994**, 57 (9), 1200-1205• DOI:
10.1021/np50111a002 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 57, No. 9, pp. 1200-1205, September 1994

(+)-(*S*)-DIHYDROAERUGINOIC ACID, AN INHIBITOR OF *SEPTORIA TRITICI* AND OTHER PHYTOPATHOGENIC FUNGI AND BACTERIA, PRODUCED BY *PSEUDOMONAS FLUORESCENS*

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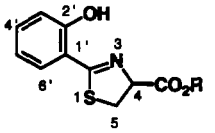
ABSTRACT.—Three antibiotics were isolated from a CH_2Cl_2 extract of the liquid culture of *Pseudomonas fluorescens* strain PFM2. Two of the antibiotics were identified as 2,4-diacetylphloroglucinol and pyoluterin. The structure elucidation, absolute stereochemistry, synthesis, and biological activities of the new antibiotic (+)-(*S*)-dihydroaeruginoic acid [**1**] are reported.

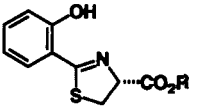
An intense public interest in the quality of our environment has stimulated a commensurate interest in the utilization of naturally occurring microorganisms as control agents of plant diseases. The genus *Pseudomonas* includes species and strains that repeatedly have been recognized as antagonists of plant pathogens (1–7). Several secondary metabolites produced by fluorescent *Pseudomonas* species are inhibitory to bacteria and fungi (8) and thus, believed to be responsible for the antagonistic profile presented by their producers. In recent years we have been involved in a study to elucidate the mechanism by which fluorescent *Pseudomonas* species inhibit wheat-leaf pathogens (9). *Pseudomonas fluorescens* (strain PFM2), isolated from wheat leaves, is antagonistic to *Septoria tritici* and also inhibits the growth of several other phytopathogenic bacteria and fungi in vitro. This characteristic of *P. fluorescens* strain PFM2, which makes it a potential biocontrol agent, encouraged us to examine the content of its antibiotic production. Inhibition of *Septoria tritici* by strain PFM2 and its major antibiotic, 2,4-diacetylphloroglucinol (10) was reported earlier (11). A second antibiotic was identified as pyoluterin, first isolated as an antifungal metabolite of *P. fluorescens* (strain Pf-5) (1). A new antibiotic, dihydroaeruginoic acid [**1**], was isolated as the second major component of strain PFM2 culture medium extract. Dihydroaeruginoic acid [**1**] contains a thiazoline ring and is related in structure to aeruginoic acid [**2**] (12) and pyochelin [**3**] (13), reported earlier from *P. fluorescens*.

RESULTS AND DISCUSSION

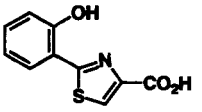
The CH_2Cl_2 crude extract was obtained from cultures of PFM2 grown for four weeks at room temperature without agitation, to minimize oxidation of metabolites (14), in a modified King's medium B (3,11). The crude extract was chromatographed on a Si gel H column to afford three fractions that inhibited growth of *Septoria tritici*. 2,4-Diacetylphloroglucinol (10), the most abundant antibiotic in the extract, was eluted from the column with 10% EtOAc in petroleum ether and identified by its physiochemical properties (11). Pyoluteorin, a second and minor active constituent of the extract, was eluted from the column with 20% EtOAc in petroleum ether and identified by



(+)-(*S*)-**1** R=H
(+)-(*S*)-**4** R=Me



(−)-(*R*)-**1** R=H
(−)-(*R*)-**4** R=Me



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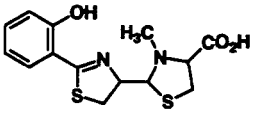

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comparison of its ms and nmr data with literature values (1). A third and major active fraction was eluted from the column with 30–40% EtOAc in petroleum ether. This third fraction consisted of almost pure (>95% by nmr and tlc) compound **1**. A broad, concentration-dependent ^{1}H-nmr signal (δ_H 7 to 9 ppm, $\Delta\ \nu_{1/2h}$=60 Hz) of this compound indicated it to be an acid. The hreims data supported this finding; the molecular ion *m/z* 223.0275 (8%, $C_{10}H_9NO_3S$, mmu error −2.8) loses a proton and CO_2 to give a major fragment ion, *m/z* 178.0329 (60%, C_9H_8NOS, mmu error 0.2). An attempt to further purify compound **1** on a semi-prep. reversed-phase hplc column, using a MeOH/H_2O eluent, afforded another pure compound [**4**] which differed from **1** in its tlc mobility [R_f of 0.8 instead of 0.2 on Si plates developed with toluene-EtOAc (1:1)] and ^{1}H-nmr spectrum (a three-proton singlet at 3.80 ppm instead of the broad acidic absorption at 7–9 ppm). On the basis of these findings we concluded that the natural acid underwent esterification with the MeOH in which it was dissolved for the hplc separation. A similar spontaneous esterification has been observed in the case of α-methylcysteine while separating the tantazoles hydrolysate by reversed-phase hplc (15). Dihydroaeruginoic acid [**1**] was later isolated and purified by flash chromatography on a Si gel H column, similar to the one described above, followed by reversed-phase hplc using a MeCN-H_2O (3:1) mixture as the mobile phase. It was found that **1** partially decomposed to salicylic acid and cysteine when chromatographed on Si gel, and slowly oxidized to aeruginoic acid [**2**] when stored as a thin film in glass vials under an air atmosphere.

The structure of **1** was deduced by spectral analysis of its methyl ester derivative, **4**, and was confirmed by total synthesis (of both **1** and **4**), which also enabled the determination of the absolute configuration of its asymmetric center. The molecular formula of the methyl ester [**4**], $C_{11}H_{11}NO_3S$, was deduced from its fabms in combination with its ^{1}H- and ^{13}C-nmr data. The ir spectrum indicated the presence of hydroxyl (3410 cm^{-1}), aromatic (1552–1592 cm^{-1}), and ester (1745 cm^{-1}) functionalities. The ^{1}H-nmr spectrum of **4** presented nine signals accounting for one exchangeable proton (δ 12.27 br s), four aromatic protons (δ 7.42 d, 7.37 dd, 7.02 d, and 6.88 dd), one methoxy group (δ 3.80 s) and a three spin (ABX) system next to a heteroatom (δ 5.35 dd, 3.68 dd, and 3.60 dd). The interpretation of an ^{1}H-COSY nmr experiment proved the aromatic protons to be part of a 1,2-disubstituted benzene ring. The ^{13}C-nmr spectrum exhibited eight sp^2 carbons (four quaternary carbons and four methines) and three aliphatic carbons (a methyl, a methylene, and a methine).

Eight of the nine signals in the ^{1}H-nmr spectrum exhibited correlations (HMQC experiment, see Experimental) with seven proton-bearing signals in the ^{13}C-nmr spectrum. Long-range ^{1}H-^{13}C correlations from the HMBC experiment allowed us to

propose for **4** a dihydroaeruginoic acid methyl ester structure. The correlations between H-4, H-5a and H-5b, and C-2 established the thiazoline ring. The correlations between H-4, H-5a, H-5b, and the methyl protons and the carboxylic carbon suggested the attachment of the carbomethoxy moiety to C-4 of the thiazoline. Correlations through two and three bonds between the aromatic protons and six of the eight sp^2 carbons (see Experimental) established the structure of the phenolic ring. The correlation between H-6′ and C-2 established the linkage between the phenolic and thiazolinic moieties.

To the best of our knowledge dihydroaeruginoic acid [**1**] is a new natural product. Indeed, the *R*-isomers of **1** and **4** have been synthesized and their antiproliferative activity on murine leukemia cells evaluated (16,17). However, the optical rotation data have not been published. We have carried out the synthesis of these compounds with L-cysteine and L-methylcysteine according to the reported methods (17,18). We found that the recently published procedure (18) for the synthesis of 2 arylthiazoles from the corresponding cyanoaryls and 2-aminothiols in methanolic phosphate buffer (pH 6) is the preferred method for the synthesis of **1** in a ca. 60% yield. For the preparation of **4**,

TABLE 1. Comparison of the Antimicrobial Activity of (*S*)-Dihydroaeruginoic Acid [(*S*)-**1**] and (*R*)-Dihydroaeruginoic Acid [(*R*)-**1**] and Concentration, by Two-Way Analysis of Variance.[a]

Microorganism	Concentration (μg/disc)	(*S*)-**1**	(*R*)-**1**	P1[b]	P2[c]
Fungi:					
Rhizoctonia solani	100	41.5 (1%)[d]	39.3 (6%)		
	200	40.0 (5%)	35.0 (17%)	0.0012	0.0039
Pythium ultimum	100	26.8 (22%)[e]	30.1 (12%)		
	200	19.6 (43%)	23.0 (33%)	0.0309	0.0004
Butrytis cinerea	100	25.6 (27%)[f]	22.5 (36%)		
	200	20.0 (43%)	19.1 (46%)	NS	0.0152
Sclerotium rolfsii.....	100	28.3 (32%)[g]	27.5 (32%)		
	200	19.7 (53%)	20.0 (52%)	NS	<0.0001
Colletotrichum	100	29.2 (8%)[h]	29.2 (8%)		
gloeosporioide.....	200	28.5 (10%)	28.8 (9%)	NS	NS
Fusarium oxysporum	100	29.5 (8%)[i]	30.6 (5%)		
	200	27.4 (15%)	28.7 (11%)	0.0134	0.0008
Septoria tritici	100	0	0		
	200	13.0	13.0	NS	
Bacteria:					
Bacillus subtilis	100	10.8	14.9		
	200	13.3	19.7	<0.0001	0.0001
Erwinia herbicola	100	6.7	8.2		
	200	8.7	10.4	0.0012	0.0003
Staphylococcus albus	100	20.2	22.0		
	200	26.2	28.2	NS	0.0002
Pseudomonas fluorescens ..	100	0	0		
(PFM2)	200	0	0		

[a]The values given for fungi, except for *S. tritici*, are the mean colony radii (in mm) and the percent of colony reduction. The values given for bacteria and *S. tritici* are the diameter (in mm) of zones of inhibition. The values represent mean of nine measurements (three different weighings×three discs per weigh).

[b]*P* value for differences between the inhibition of the microorganism growth by [(*S*)[**1**] and [(*R*)-**1**].

[c]*P* value for differences between concentrations.

[d]Mean colony radius of control: 42.0 mm.

[e]Mean colony radius of control: 34.3 mm.

[f]Mean colony radius of control: 35.3 mm.

[g]Mean colony radius of control: 41.7 mm.

[h]Mean colony radius of control: 31.7 mm.

[i]Mean colony radius of control: 32.3 mm.

in ca. 75% yield, we have used the method described by Elliot *et al.* (17), which involves the condensation of ethyl 2-hydroxybenzimidate with cysteine methyl ester HCl in dry MeOH. The latter method, when applied to the preparation of **1**, yielded a mixture of compounds **1** and **4**. Comparison of the optical rotations of the natural product [**1**, $[\alpha]^{27}D$ +47° (c=0.10, $CHCl_3$)] and its methyl ester, [**4**, $[\alpha]^{25}D$ +11.3° (c=0.15, MeOH)], with the synthetic compound from L-cysteine [**1**, $[\alpha]^{27}D$ −49° (c=0.23, $CHCl_3$)] and its methyl ester [**4**, $[\alpha]^{25}D$ −14.7° (c=0.27, MeOH)] suggested the natural product to be of the *S*-absolute stereochemistry at C-4. To confirm this suggestion the same synthesis was carried out with D-cysteine and its methyl ester. The optical rotation values of these synthetic compounds were similar to those of the natural product [**1**, $[\alpha]^{27}D$ +46° (c=0.02, $CHCl_3$)] and its methyl ester derivative [**4**, $[\alpha]^{25}D$ +14.3° (c=0.31, MeOH)]. On the basis of the results described above the structure of compound **1** was assigned as (+)-(*S*)-dihydroaeruginoic acid.

The antimicrobial activity of (*S*)- and (*R*)-**1** against a selection of phytopathogenic fungi and bacteria (see Table 1) was compared. The unnatural isomer (*R*)-**1** was more active than the natural (*S*)-**1** against *Rhizoctonia solani, Bacillus subtilis,* and *Erwinia herbicola*. The natural isomer (*S*)-**1** was more active than the unnatural (*R*)-1 against *Pythium ultimum* and *Fusarium oxysporum*. Similar inhibition properties were observed for both compounds against *Butrytis cinerea, Sclerotium rolfsii, Colletotrichum gleosporioide,* and *Staphylococcus albus*, and both compounds showed no inhibitory activity against *P. fluorescens* (strain PFM2) itself.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ft-ir spectra were recorded on a Nicolet Ft-ir instrument in $CHCl_3$ or neat. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Uv spectra were recorded on a Kontron 931 plus spectrophotometer. Nmr spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^1H nmr and 125.76 MHz for ^{13}C nmr. ^1H-, ^{13}C-, DEPT, COSY-45, HMQC, and HMBC nmr spectra were recorded using standard Bruker pulse sequences. Lrms were obtained with a VG Tritech TS-250 mass spectrometer equipped with an 11-250J data system; the hrms was recorded on a Finnigan MAT 711 mass spectrometer. Hplc separations were performed with an Applied Biosystem Inc. instrument equipped with two model 150 pumps and a 893 programable uv detector.

EXTRACTION AND ISOLATION.—For antibiotic production, *P. fluorescens* strain PFM2 was cultured in a modified liquid King's medium B (11) without agitation to minimize oxidation of metabolites. This medium (GGP) consisted of 30 ml glycerol, 10 g proteose peptone No. 3 (Difco), and 0.5 g each of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ per liter of distilled H_2O. The crude material (826 mg) extracted with CH_2Cl_2 from liquid medium (10 liters) in which *P. fluorescens* strain PFM2 was cultured (11), was loaded onto a vacuum column (Merck Si gel, 5 g, packed into a 2 cm i.d.×30 cm high, sintered glass funnel; vacuum by H_2O aspirator) and eluted in petroleum ether with an increasing EtOAc step gradient to yield 12 fractions (50 ml each). The fractions were assayed for antifungal activity against *S. tritici* at a concentration of 0.2 mg/disc. Fractions 2, 3, 4, and 5 were the most active, with inhibition zones of 26, 10, 14, and 14 mm, respectively. Fraction 2 was crystallized from MeOH/H_2O as needles. The pure compound was identified as 2,4-diacetylphloroglucinol as previously reported by two of the present authors (11). The third fraction (9.3 mg) contained one major component which was purified on a semi-prep. hplc column (YMC AM-324, ODS, 120A, 10×300 mm) using MeOH-H_2O (3:1) as the eluent (3 ml/min). The major component of this fraction (3.1 mg, R_t 10.0 min) was identified as pyoluterin. The combined material (10.4 mg), from the fourth and fifth fractions, was initially dissolved in MeOH and subjected to the same semi-prep. hplc column using MeOH-H_2O (3:1) as the eluent (3 ml/min) to afford compound **4** (5.2 mg, R_t 11.5 min). In later purifications, these fractions (52 mg) were dissolved in MeCN and eluted from the same column using MeCN-H_2O (3:1) as the eluent (2.5 ml/min) to afford compound **1** (27.1 mg, R_t 15.2 min).

(+)-(S)-Dihydroaeruginoic acid [**1**].—$[\alpha]^{27}D$ +47° (c=0.10, $CHCl_3$); uv λ max (MeCN) 315 (ε 6670), 252 (15800), 214 (31730) nm; ir ν max (neat) 3000, 1755, 1610, 1515, 1320, 1240, 1070, 950, 800, and 750 cm^{-1}; ^1H nmr ($CDCl_3$) δ 5.41 (1H, dd, J=8.1 and 9.3 Hz, H-4), 7.10 (1H, br s, CO_2H on C-4), 3.65 (1H, dd, J=9.3 and 11.4 Hz, H-5a), 3.69 (1H, dd, J=8.1 and 11.4 Hz, H-5b), 12.10 (1H, br s, OH on C-2′), 7.02 (1H, dd, J=1.0 and 8.3 Hz, H-3′), 7.39 (1H, ddd, J=1.6, 7.5, and 8.3 Hz, H-4′), 6.89 (1H, ddd, J=1.0, 7.5, and 7.8 Hz, H-5′), 7.42 (1H, dd, J=1.6 and 7.8 Hz, H-6′); ^{13}C-nmr ($CDCl_3$) δ (multiplicity,

carbon position, proton HMBC correlations) 174.18 (s, C-2, H-4, H-5a, H-5b and H-6'), 76.19 (d, C-4, H-5a and H-5b), 175.08 (s, CO_2H on C-4, H-4, H-5a and H-5b), 33.55 (t, C-5, H-4), 115.80 (s, C-1', H-3' and H-5'), 159.08 (s, C-2', H-3', H-4', and H-6'), 117.32 (d, C-3', H-5'), 133.74 (d, C-4', H-6'), 119.02 (d, C-5', H-3'), 130.78 (d, C-6', H-4'); hreims (70 eV) *m/z* 223.0275 M^+, 8%, calcd for $C_{10}H_9NO_3S$, mmu error −2.8, 178.0329 60%, calcd for C_9H_8NOS, mmu error 0.2, and 119.0382 11%, calcd for C_7H_5NO, mmu error 1.1.

(+)-(S)-Methyl dihydroaeruginoic acid [**4**].—$[\alpha]^{25}D$ +11.3° (*c*=0.15, MeOH); uv λ max (MeOH) 316 (ε 4700), 252 (12000), 214 (34760) nm; ir ν max ($CHCl_3$) 3410, 2970, 1745, 1623, 1592, 1552, 1255, 1220, and 1008 cm^{-1}; ^1H nmr ($CDCl_3$) δ 5.35 (1H, dd, *J*=8.0 and 9.5 Hz, H-4), 3.80 (1H, s, CO_2CH_3 on C-4), 3.60 (1H, dd, *J*=9.5 and 11.0 Hz, H-5a), 3.68 (1H, dd, *J*=8.0 and 11.0 Hz, H-5b), 12.25 (1H, br s, OH on C-2'), 7.02 (1H, dd, *J*=1.0 and 8.5 Hz, H-3'), 7.37 (1H, ddd, *J*=2.0, 7.5 and 8.5 Hz, H-4'), 6.88 (1H, ddd, *J*=1.0, 7.5, and 8.0 Hz, H-5'), 7.42 (1H, dd, *J*=2.0 and 8.0 Hz, H-6'); ^{13}C nmr ($CDCl_3$) δ (multiplicity, carbon position, proton HMBC correlations) 174.72 (s, C-2, H-4, H-5a, H-5b, and H-6'), 76.72 (d, C-4, H-5a, and H-5b), 170.63 (s, CO_2CH_3 on C-4, H-4, H-5a, H-5b, and CO_2CH_3 on C-4), 52.89 (q, CO_2CH_3 on C-4), 33.67 (t, C-5, H-4), 115.98 (s, C-1', H-3', and H-5'), 159.17 (s, C-2', H-3', H-4', and H-6'), 117.28 (d, C-3', H-5'), 133.58 (d, C-4', H-6'), 118.98 (d, C-5', H-3'), 131.10 (d, C-6', H-4'); ms (fab thioglycerol/glycerol) *m/z* [MH^+] 238, 204, 178, 149, 133, 120.

SYNTHESIS OF DIHYDROAERUGINOIC ACID [**1**].—L- and D-Cysteine hydrochlorides were reacted with *o*-cyanophenol in MeOH-0.1 M phosphate buffer (pH 6) (1:1) at room temperature for 4 days, to afford (*R*)- and (*S*)-dihydroaeruginoic acids according to the method of Hoveyder *et al.* (18), in yields of 60% and 57%, respectively. (*R*)- and (*S*)- dihydroaeruginoic acids [(*R*)-**1** and (*S*)-**1**] exhibited ms, ir, uv, and nmr properties similar to natural **1**. (*R*)-Dihydroaeruginoic acid [(*R*)-**1**] presented a negative optical rotation value, $[\alpha]^{27}D$ −49° (*c*=0.23, $CHCl_3$), while (*S*)-dihydroaeruginoic acid [(*S*)-**1**] exhibited a positive optical rotation value, $[\alpha]^{27}D$ +46° (*c*=0.02, $CHCl_3$).

SYNTHESIS OF METHYL DIHYDROAERUGINOATE [**4**].—Ethyl 2-hydroxybenzimidate hydrochloride was prepared by an established method (19). (*R*)- and (*S*)-Methyl dihydroaeruginoate were prepared by condensation of L- and D-cysteine hydrochloride methyl esters with ethyl 2-hydroxybenzimidate according to the method of Elliot *et al.* (16,17), in yields of 70% and 75%, respectively. (*R*)- and (*S*)-Methyl dihydroaeruginoate [(*R*)-**4** and (*S*)-**4**] presented ms, ir, uv, and nmr properties similar to **4** obtained from the natural product. (*R*)-Methyl dihydroaeruginoate [(*R*)-**4**] exhibited a negative optical rotation value, $[\alpha]^{25}D$ −14.7° (*c*=0.27, MeOH), while (*S*)-methyl dihydroaeruginoate [(*S*)-**4**] exhibited a positive optical rotation value, $[\alpha]^{25}D$ +14.3° (*c*=0.31, MeOH).

TEST MICROORGANISMS AND MEDIA.—The culture of *P. fluorescens* (strain PFM2) was obtained from stock maintained at the Plant Sciences and Water Conservation Laboratory, USDA, ARS, Stillwater, OK. Other cultures of fungi and bacteria were obtained from stocks maintained at the Department of Plant Protection and Inspection Clinical Laboratory, Ministry of Agriculture, Bet-Dagan, Israel, as follows: *Septoria tritici* (strain ISR-SE93), *Fusarium oxysporum* (strain Fo0593-22), *Sclerotium rolfsii* (strain RG-1984), *Pythium ultimum* (strain RG-5601), *Rhizoctonia solani* (strain DL5588), *Colletotrichum gloeosporioides* (strain DL5605), *Butrytis cinerea* (strain DL6050), *Erwinia herbicola* (strain SM-824), *Bacillus subtilis* (strain Bs1091-1), and *Staphylococcus albus* (strain Sal1091-4).

Septoria tritici was maintained on a malt agar medium (MA) containing 4 g each of malt extract, yeast extract, and sucrose, and 20 g of agar per liter of distilled H_2O. One-fourth-strength Potato Dextrose Agar (PDA, Difco) was used as substrate for maintaining and testing the other fungi. Bacteria were maintained and tested on Luria agar medium (LA) containing 10 g of triptone (Difco), 5 g yeast extract (Difco), 5 g of sodium chloride, and 16 g of bacto agar (Difco) per liter of distilled H_2O.

INHIBITION ASSAYS.—The antibiotic effects of the crude culture extract, the semipure fractions, the pure isolates, and the synthetic compounds on *S. tritici* and the other fungi and bacteria were determined by the paper-disc agar-diffusion bioassay. For each of the pure compounds, (*R*)-**1** and (*S*)-**1**, three separately weighed solutions were prepared. The appropriate volume of stock solution was absorbed on 6-mm paper disc. Three replicates from each of the stock solutions (nine total) were tested per tested microorganism. In the case of fungi that grow by hyphal extension, a mycelium in an agar plug (5 mm diameter) from the edge of the growing culture was placed at the center of the petri dish. A paper disc preabsorbed with Me_2CO solution of the antibiotic and a control disc, prepared with Me_2CO, were placed from both sides and at equal distances (30 mm) from the agar plug in the center of the dish. A few days later (2–6 days, depending on the growth rate of each fungus), the radii of each colony were measured along a line congruent to an imaginary line that connected the centers of the two paper discs. Reduction in radial growth of the colony was calculated as the difference in the radii of the colony near the antibiotic-laden disc and near the antibiotic-free disc,

divided by the later radius, and expressed as percent. In the case of *S. tritici* and bacteria the antimicrobial activity is presented as zone of inhibition around the paper disc (6 mm) absorbed with the antibiotic.

ACKNOWLEDGMENTS

The authors thank Cindy Warfield, Genia Elkind, and Bella Kuchuk for their excellent technical assistance.

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Received 28 September 1993