3′-Amino-3′-deoxyadenosine, an Antitumor Agent from *Helminthosporium* sp.[1]

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An antitumor agent from *Helminthosporium* sp. culture filtrates has been obtained in pure form and identified as 3′-amino-3′-deoxyadenosine.

Ammann and Safferman observed that culture filtrates from a *Helminthosporium* sp. No. 215 showed strong antimitotic effects in an onion root tip test and was also active against tumors in mice.[2] A crude extract of mycelium from the *Helminthosporium* exhibited antitumor activity without significant toxicity against four ascitic tumors of mice when administered intraperitoneally.[3] Since the crude extract showed no antimicrobial properties, production batches, and purification steps in the early phases of the work were assayed in mice against the ascitic form of Ehrlich Lettré hyperdiploid carcinoma.[4] The bulk of the antitumor principle was found in the broth from which it could not be extracted with butanol. The active compound was retained by strongly basic and strongly acidic but not by weakly basic ion exchange resin columns. Absorption of bases from the broth on a sulfonated polystyrene ion exchange column followed by elution with aqueous ammonia produced a brown gum 10 times more effective than the crude extract against tumors and which absorbed strongly at 260 mμ. The amphoteric properties and ultraviolet spectrum of the gum suggested the presence of a purine nucleus. Paper chromatography of the gum in dilute ammonia revealed a band opaque to transmitted ultraviolet light, R_f about 0.5. This band from one paper contained enough material to inhibit tumor growth in five mice. All subsequent crude and partially purified materials were rapidly and conveniently assayed by paper chromatography. A second purification step was readily effected on a cellulose column with dilute ammonia. The bulk of the inert material moved with the solvent front; a second ten-fold concentration of activity was thus achieved The average yield was 65 mg. per liter of broth.

In pure form the active product crystallized readily and was only slightly soluble in water. It had a molecular formula of $C_{10}H_{14}N_6O_3$ and a search of the literature suggested that it was identical with 3′-amino-3′-deoxyadenosine prepared by a lengthy chemical synthesis from adenine and D-xylose.[5] The detailed comparison of properties (Table I) confirmed this. Cleavage of the glycoside linkage produced adenine and 3-amino-3-deoxyribose identical with authentic specimens.

TABLE I

3′-AMINO-3′-DEOXYADENOSINE

	Synthetic		From *Helminthosporium* sp.
M.p.	265–267° dec.[a]		271–273° dec. (hot stage)
$[\alpha]^{25}D$	−40° (0.4% in DMF)[b]		−37° (2% in 0.1 *N* HCl)
$\lambda_{max}^{H_2O}$	259 mμ (ε = 15,000)[b]		260 mμ (ε = 17,300)
λ_{max}^{KBr}	3.00, 3.17	λ_{max}^{Nujol}	3.1, 3.3
	6.00, 6.22, 6.37		6.1, 6.3, 6.48
	9.13, 9.28, 9.66[a]		9.2, 9.4, 9.7
R_{ad} in 5% Na_2HPO_4 = 1.25[a]			Same
	Phthalimido derivative		
M.p.	228–230°[b]		240.5–242.5° (hot stage)
$[\alpha]^{25}D$	−175° (0.6% in C_2H_5OH)[b]		−188° + 8° (0.7% in C_2H_5OH)

[a] E. J. Reist and B. R. Baker, *J. Org. Chem.*, **23**, 1083 (1958). [b] See ref. 5.

In streak dilution assay at 500 γ/ml. pure 3′-amino-3′-deoxyadenosine produced almost complete inhibition of growth of the two yeasts tested but had no activity against several bacteria and filamentous fungi. The LD_{50} in mice was 28 mg./kg. by intraperitoneal injection.[6] Antitumor studies in mice are in progress.

Experimental

Preparation.—Yeast extract–Cerelose slants of the *Helminthosporium* sp. were washed down and each used to inoculate two 250-ml. Erlenmeyer flasks containing 100 ml. of 1% yeast extract–1% Cerelose broth (*p*H 6.5). These inoculum flasks were shaken at 215 r.p.m. at 28° for 5 days; those with black granular growth were checked for freedom from bacterial contamination[7] and each used to inoculate four 2-l. production flasks which contained 500 ml. of 1% yeast extract–1% cerelose broth (*p*H 6.5–6.8) and 1.25 g. of calcium carbonate. The production flasks were shaken at

(1) The U. S. Public Health Service (CY 4794) supported this investigation.

(2) C. A. Ammann and R. S. Safferman, *Antibiotics & Chemotherapy*, **8**, 1 (1958).

(3) L. H. Pugh, H. A. Lechevalier, and M. Solotorovsky, *Antibiotics & Chemotherapy*, in press.

(4) L. H. Pugh, N. N. Gerber, H. A. Lechevalier, and M. Solotorovsky, to be published.

(5) B. R. Baker, R. E. Schaub, and H. M. Kissman, *J. Am. Chem. Soc.*, **77**, 5911 (1955).

(6) L. H. Pugh, private communication.

(7) Bacteria could be present even though the broth was clear; we routinely streaked a few drops of broth on nutrient agar plates. Incubation at 37° for 2 days revealed any bacteria; the inoculum flasks meanwhile were stored at 5°.

100 r.p.m. for 7 days, at which time the *p*H was 8 or higher. The contents were filtered by gravity then the mycelium pressed down and sucked very dry on a Buchner funnel. The combined broths should be absolutely clear; cloudiness indicates bacterial contamination. The mycelium was extracted twice in a Waring blender using each time 3 ml. of distilled water per gram. The yields from broth, which always contained at least 90% of the active principle, varied from 46–102 μg./ml. (average 65 μg./ml.) by paper chromatographic assay.

Combined broth and mycelium extracts (10–25 l.) were acidified to pH 5.0–5.5 with 10% hydrochloric acid (about 50 ml. per 2 l.) degassed in 2 l. portions with a water pump for 1/2 hour then applied to an 800 ml. volume (about 7.5 × 21 cm.) ion-exchange resin column (IR 120 CP about 5% crosslinked) in the hydrogen form. The rate of application was 2–3 l./hr. The column was then washed with 4 l. of distilled water and eluted with 1.4% ammonium hydroxide. Three 2-l. eluate fractions were collected when the effluent pH became greater than 7 and assayed by paper chromatography. Fractions which contained at least 50 mg. of product and whose optical density ratio (see assay, next section) was at least 2 were combined, reduced to 1/10 volume in a flash evaporator, seeded, and refrigerated 1 week. Filtration usually furnished tan crystalline product, up to 3/4 of that present in the solution as indicated by paper chromatographic assay. The filtrate was reduced to $^1/_4$–$^1/_2$ volume and applied to a 600-g. packed cellulose column (4.5 × 81 cm.) which had been washed with 2.8% ammonium hydroxide until the optical density of the eluate at 260 mμ was 0.2 or less. Elution was also with 2.8% aqueous ammonia; 20-ml. fractions were collected. The first several dark brown fractions were discarded; later tan and colorless fractions whose optical density ratio was 2 or more were combined and reduced to dryness in the flash evaporator. The residue was dissolved in 1% acetic acid and the resulting solution (not more than 25 ml.) was made alkaline with concentrated ammonia, cooled several days, and filtered. The over-all recovery of aminonucleoside was essentially 100%.

For further purification, crude material was dissolved in 100 parts of dilute acetic acid. The mixture was filtered and made strongly basic with ammonium hydroxide; nearly white, fine needles (85% recovery) were obtained after 3 days in the cold.

Paper Chromatographic Assay.—One-milliliter aliquots of broth, mycelium extract or column eluate were applied as a line to Whatman #1 paper, 4 in. × 18 in., which had been previously washed by descending chromatography with 2.8% aqueous ammonia and dried. The sample was chromatographed by the descending method with 2.8% ammonium hydroxide for 2–3 hours, dried, and the nucleoside band at R_f about 0.5 located by its opacity to ultraviolet light of 250 mμ. The band was cut out and eluted by descending chromatography overnight with water in a desiccator.[8] The eluate was diluted to 10 ml. and its ultraviolet light absorbtion measured from 220–300 mμ. The yield of nucleoside was calculated from the optical density at 260 mμ and the previously determined $E^{1\%}_{1\,cm.}$ of pure 3′-amino-3′-deoxyadenosine which is 650. Many other components of the broth absorb ultraviolet light with the result that impure fractions may have gradually decreasing absorbtion from 220–300 mμ with a slight peak at 260 mμ. Calculations based on such spectra are invalid; all fractions with an optical density ratio (optical density at 260 mμ/optical density at 230 mμ) of less than 2 were discarded. For pure product the ratio was 4–5.

Properties.—The pure aminonucleoside is soluble (10 mg./1 ml.) in hot water, hot methanol, dimethylformamide, and dimethyl sulfoxide; slightly soluble in hot ethanol, hot chloroform, and hot pyridine; insoluble in butanol, acetone, tetrahydrofuran, ethyl acetate, and butyl acetate hot or cold. It gave no color with ferric chloride, ninhydrin or diazotized sulfanilic acid, no precipitate with 2,4-dinitrophenylhydrazone and decolorized permanganate slowly. On paper it did not reduce silver nitrate but gave a faint ninhydrin test after prolonged heating. The analytical sample precipitated from a dilute acetic acid solution by ammonia melted at 171–173° with decomposition after vacuum drying at 100°.

Anal. Calcd. for $C_{10}H_{14}N_6O_3$: C, 45.11; H, 5.30; N, 31.57. Found: C, 44.90; H, 5.31; N, 32.42.[9]

The dipicrate derivative melted at 222–224° with decomposition after vacuum drying at 100°.

Anal. Calcd. for $C_{22}H_{20}N_{12}O_{17}$: C, 36.45; H, 2.78; N, 23.20; M. W. 724.5. Found: C, 36.43; H, 2.97; N, 23.35[9]; M.W. 732 (spectrophotometric[10]).

After cleavage of the aminonucleoside[5] the addition of picric acid furnished adenine picrate identical in m.p. and mixed m.p. with an authentic sample. Milder cleavage[11] produced adenine hydrochloride identical in m.p. and R_f value in five solvent systems with authentic material and a reducing sugar. This reducing sugar could be isolated in milligram amounts by paper chromatography of the hydrolysis mixture (0.5 ml. on each of four 7 in. times 18 in. Whatman #3 papers which had been previously washed with water) for 24 hr. in butanol–acetic acid–water (4:1:5) (upper layer). The sugar bands were located by spraying a narrow marker strip with aniline oxalate, cut out, and eluted with water.[8] Removal of the solvent in a vacuum oven gave needles of 3-amino-3-deoxyribose hydrochloride m.p. 154–158° dec. identical with that prepared in a similar manner from 6-dimethylamino-β-D-(3-amino-3-deoxyribofuranosyl)purine.[12]

Antimicrobial Assay.—Pure 3′-amino-3′-deoxyadenosine in warm 10% aqueous dimethylsulfoxide was assayed by the streak dilution method at 500 and 100 γ/ml. Solvent controls were also prepared. The fungi on Sabouraud glucose medium were incubated at 30° for 48 hr. and the bacteria on Difco nutrient agar were grown at 37° for 24 hr. There was almost complete inhibition of *Cryptococcus neoformans* 4806 and *Candida albicans* (Drouhet-Souche Normande) at 500 γ/ml. and a slight reduction of growth at 100 γ/ml. There was no inhibition of *Staphylococcus aureus* strains LeCompte and Valentin, *Escherichia coli* strains A. V. 2 and Monod, *Pseudomonas aeruginosa* Ingant, *Enterococcus* Fanjou, *Sarcina lutea* A.T.C.C. 9341, *Bacillus brevis* 5122 1P, *Bacillus cereus* A.T.C.C. 10702, *Bacillus subtilis* strains CHP, and A.T.C.C. 6633, *Aspergillus niger* 4823, *Penicillium* sp. 4847, *Trichophyton mentagrophytes* 4805.

There was partial inhibition only at 100 γ/ml. of yeast protoplasts from *Saccharomyces cerevisiae* LK2G12.

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(8) For the exact method see E. Lederer and M. Lederer, "Chromatography, a Review of Principles and Applications," 2nd ed., Elsevier Publishing Co., New York, 1957, p. 139.

(9) George Robertson, Florham Park, N. J.

(10) K. C. Cunningham, W. Dawson, and F. S. Spring, *J. Chem. Soc.*, 2305 (1951).

(11) H. R. Bently, K. C. Cunningham, and F. S. Spring, *J. Chem. Soc.*, 2301 (1951).

(12) Kindly provided by Dr. M. J. Weiss, Lederle Division, American Cyanamid Co.