[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Synthesis and Biological Activity of Some 2-Amino-6-(substituted)-purines

BY EDWARD O. LEONARD, CHARLES G. SKINNER, EDWIN M. LANSFORD, JR., AND WILLIAM SHIVE

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Five new 2-amino-6-(substituted)-aminopurines were prepared by a thermal condensation of 2-amino-6-methylthiopurine and the appropriate amine, and all were found to augment the growth inhibition of 2,4-diamino-6,7-diphenylpteridine upon *Lactobacillus arabinosus*. Some of the diaminopurine derivatives stimulated the rate of lettuce seed germination; however, they were somewhat less active than the corresponding 6-(substituted)-aminopurines.

Several 6-(substituted)-purines have been found to possess activity in many different biological systems; for example, they promote cell division in tobacco callus tissue,[1] inhibit hydra tentacle regeneration,[2] increase the rate of lettuce seed germination,[3,4] stimulate moss bud formation,[5] augment the growth inhibition of 2,4-diamino-6,7-diphenylpteridine upon *Lactobacillus arabinosus*,[6] and recently have been observed to act synergistically with gibberellin in promoting seed germination.[7] While the group substituted in the 6-position may be modified considerably with retention of biological activity, other changes in the purine nucleus have resulted in compounds which are inactive in most of these test systems.[8]

In the present investigation, a number of the more effective 6-(substituted)-purine analogs were modified by introducing an amino group in the 2-position. Five new 2-amino-6-(substituted)-aminopurines possessing some of the substituents which conferred potent biological activity in the adenine series were prepared and found to possess activity in the pteridine-inhibited bacterial system, and three of these 2,6-disubstituted purine derivatives possessed appreciable activity in stimulating the rate of lettuce seed germination.

These compounds were synthesized by a condensation of the appropriate amine with 2-amino-6-methylthiopurine in a sealed bomb at about 150°.[9] In general, the products were more difficult to separate from these reaction mixtures than were the corresponding monosubstituted purine derivatives, and the over-all yields were appreciably lower. The ultraviolet absorption spectra of all of these 2-amino-6-(substituted)-aminopurines were essentially the same, with two λ_{max} at about 282–284 mμ and about 248–250 mμ as determined in 95% ethyl alcohol.[9] Bioautographs of the compounds using the bacterial assay procedure previously reported[6] showed only a single zone of biological activity. The starting material, 2-amino-6-methylthiopurine, did not possess any inhibitory properties in this biological system.

In the pteridine-inhibited *L. arabinosus* assay system, the activities of the 2-amino-6-(substituted)-aminopurines were greater than the corresponding 6-(substituted)-aminopurines; the relative activities of the new analogs are shown in Table I. This assay system is an adaptation of a previously reported microbiological assay for thymidine[10] in which 6-(substituted)-aminopurines were observed to augment the growth inhibition by 2,4-diamino-6,7-diphenylpteridine in the absence of thymidine. 6-(2-Furfuryl)-aminopurine (Kinetin) gave augmented zones of inhibition of 28 and 33 mm. (20 and 40 γ/disk, respectively); the corresponding 2,6-disubstituted purine analog, 2-amino-6-(2-furfuryl)-aminopurine, gave values of 33 and 39 mm., respectively, at the same concentrations. With the exception of the benzyl derivative, 2-amino-6-benzylaminopurine, all of the other 2,6-disubstituted purine analogs (Table I) were more inhibitory than the corresponding adenine derivatives. The two analogous benzyl-aminopurine derivatives produced essentially the same inhibitory response in the *L. arabinosus* paper disk assay. In this assay the toxicity of each of these 2-amino-6-(substituted)-aminopurine derivatives was reversed by thymidine, as in the case of the corresponding adenine analogs.

TABLE I

SOME BIOLOGICAL ACTIVITIES OF 2-AMINO-6-(SUBSTITUTED)-AMINOPURINES

Substituted group	*Lactobacillus arabinosus* Diameter of growth inhibition zone, mm.[a] Weight of cmpd./disk 20 γ	40 γ	Stimulation of rate of lettuce seed germination Percentage of seed germinated after pretreatment in a 30 γ/ml. solution of the purine[b] Time 24 hours Supplement None	100γ Gib.	48 hours Supplement None	100γ Gib.
Water control	..	..	0	20	2	55
Benzyl-	30	37	19	65	86	95
2-Phenethyl-	33	38	3	50	35	90
2-Furfuryl-	33	39	5	25	10	53
β-Pyridylmethyl-	25	30	1	20	10	51
γ-Pyridylmethyl-	20	23	11	55	65	92

[a] This assay procedure is described in ref. 6. [b] This assay procedure is described in ref. 14; Gib = gibberellin; data given are an average of 3 experiments using 100 or more seeds for each assay.

In contrast to the generally enhanced activity of the diaminopurine analogs as compared to the adenine derivatives in the bacterial assay system

(1) C. O. Miller, F. Skoog, F. S. Okumura, M. H. Von Saltza and F. M. Strong, THIS JOURNAL, **78**, 1375 (1956).

(2) C. G. Skinner, W. Shive, R. G. Ham, D. C. Fitzgerald and R. E. Eakin, *ibid.*, **78**, 5097 (1956).

(3) C. O. Miller, *Plant Physiol.*, **31**, 318 (1956).

(4) C. G. Skinner, R. J. Claybrook, F. D. Talbert and W. Shive, *ibid.*, **32**, 117 (1957).

(5) B. S. Gorton, C. G. Skinner and R. E. Eakin, *Arch. Biochem. Biophys.*, **66**, 493 (1957).

(6) E. M. Lansford, Jr., C. G. Skinner and W. Shive, *ibid.*, **73**, 191 (1958).

(7) C. G. Skinner and W. Shive, *ibid.*, **74**, 283 (1958).

(8) C. G. Skinner and W. Shive, *Plant Physiol.*, **32**, 500 (1957).

(9) J. A. Montgomery and L. B. Holum, THIS JOURNAL, **80**, 404 (1958).

(10) E. M. Lansford, Jr., W. M. Harding and W. Shive, *Arch. Biochem. Biophys.*, **73**, 180 (1958).

discussed above, several of these 2-amino-6-(substituted)-aminopurines were appreciably less stimulatory than the corresponding 6-(substituted)-aminopurine derivatives in stimulating the rate of seed germination. Thus there appears to be a greater specificity of structure for induction of germination activity within the diaminopurine series, as contrasted to the adenine series, with respect to the substituent group in the 6-position. Of the compounds studied, the benzyl- and γ-pyridylmethyl- groupings are the most potent. Pretreatment of Early Curled Simpson lettuce seed in a solution containing 30 γ/ml. of 2-amino-6-benzylaminopurine hydrochloride or 2-amino-6-γ-pyridylmethylaminopurine, followed by germination in the dark at 30° for 48 hours on filter paper wet with water, resulted in 86 and 65% germination, respectively, as compared to a water control of only 2%. The phenylethyl-, furfuryl- and β-pyridylmethyldiaminopurine derivatives gave only 35, 10 and 10% germination, respectively, under identical experimental conditions. The adenine derivatives corresponding to the latter three compounds are significantly more stimulatory than the 2,6-diaminopurine analogs even at lower concentrations (3–10 γ/ml.). Further, 2-amino-6-benzylthiopurine was inactive in stimulating the rate of lettuce seed germination, whereas the corresponding 6-benzylthiopurine did induce germination activity.[11]

As indicated in Table I, the 6-benzyl-, 6-(2-phenylethyl)- and 6-(γ-pyridylmethyl)-diaminopurine derivatives are synergistic with gibberellin in stimulating the rate of lettuce seed germination. The data recorded in the table include the synergistic effect obtained with a mixture of 30 γ/ml. of the diaminopurine compounds and 100 γ/ml. of gibberellin; however, if the level of the purine analogs is decreased to 10 γ/ml. an appreciable synergistic stimulation of germination is still observed. For example, lettuce seed treated with the 2-amino-6-(substituted)-aminopurine derivatives given above, at a concentration of 10 γ/ml., were 0, 1 and 8% germinated, respectively, after 24 hours; whereas, a 10 γ/ml. solution of these analogs supplemented with 100 γ/ml. of gibberellin induced 84, 60 and 64% germination, respectively, after 24 hours in the dark at 30°. Gibberellin alone at this concentration under identical conditions gave a value of 18%, and seeds presoaked in water alone were essentially non-germinated under these testing conditions.

These 2-amino-6-(substituted)-purine derivatives constitute a new class of purine compounds which possess biological activities in several different types of assay systems comparable to those associated with 6-(substituted)-purines.

Experimental[12]

2-Amino-6-methylthiopurine.—One gram of 6-thioguanine was dissolved in one equivalent of 1 *N* sodium hydroxide with warming, after which about 25 ml. of water was added and then the solution was allowed to cool to room temperature. One equivalent of methyl iodide was added to the well-stirred solution during one hour, and the reaction mixture was allowed to stir an additional two hours at room temperature. After standing in the refrigerator overnight, a precipitate formed which was recovered and dried *in vacuo* over phosphorus pentoxide to yield 0.98 g. of material, m.p. 237–241° dec.[13]

2-Amino-6-benzylthiopurine.—Using the same preparative procedure described above for the methylthio derivative, 0.2 g. of 6-thioguanine after reaction with benzyl chloride gave 0.28 g. of product, m.p. 207–209°.

Anal. Calcd. for $C_{12}H_{11}N_5S$: N, 27.22. Found: N, 27.13.

2-Amino-6-benzylaminopurine Hydrochloride.—A mixture of 500 mg. of 6-methylthioguanine and 3 ml. of benzylamine was placed in a micro Carius tube, sealed under nitrogen, and heated at about 150° for 14 hours. After cooling, the bomb was opened, the reaction mixture was taken up in 15 ml. of ether, and the resulting solution was cooled in an ice-bath. Upon filtering the mixture, there was recovered 155 mg. of impure material, m.p. 244–260° dec. The filtrate was reduced to dryness, taken up in 95% ethyl alcohol, and passed through a 20 × 1.2 cm. Amberlite IRC-50 resin column (hydrogen form). The column was successively eluted with 150 ml. each of 0.01, 0.03 and 0.05 *N* hydrochloric acid, and an ultraviolet absorption spectrum of the several eluates indicated that the purine derivative was in the last fraction. This fraction was taken to dryness *in vacuo*, and the residue was freed of excess acid by the repeated addition and evaporation of ethanol. The residue finally was taken up in ethanol, decolorized with charcoal, and placed in the refrigerator to yield 64 mg. of product, m.p. 230–233° dec.

Anal. Calcd. for $C_{12}H_{12}N_6 \cdot HCl$: C, 52.06; H, 4.73; N, 30.37. Found: C, 52.20; H, 4.91; N, 30.28.

2-Amino-6-(2-furfuryl)-aminopurine.—Using the condensation procedure described above, 250 mg. of 6-methylthioguanine and 1.5 ml. of furfurylamine were heated at 150° for 12 hours. The reaction mixture was taken up in 20 ml. of cold ether, and the resulting brown oil was removed. The ether phase was reduced in volume and cooled in the refrigerator overnight to yield 77 mg. of product, m.p. 206–208°.

Anal. Calcd. for $C_{10}H_{10}N_6O$: C, 52.16; H, 4.38; N, 36.51. Found: C, 52.41; H, 4.48; N, 36.31.

2-Amino-6-(β-pyridylmethyl)-aminopurine.—Using the same general procedure described for the benzyl analog, 500 mg. of 6-methylthioguanine and 3 ml. of β-pyridylmethylamine were heated to 150° for 15 hours. The reaction mixture was reduced to dryness *in vacuo*, and the residue was taken up in 20 ml. of ether. Upon cooling, a precipitate formed, which was taken up in 95% ethyl alcohol, decolorized with charcoal, and reduced to dryness again. The resulting residue was recrystallized from acetone to yield 185 mg. of product, m.p. 251–253°.

Anal. Calcd. for $C_{11}H_{11}N_7$: C, 54.76; H, 4.60. Found: C, 54.59; H, 5.05.

2-Amino-6-(γ-pyridylmethyl)-aminopurine.—A mixture of 500 mg. of 6-methylthioguanine and 3 ml. of γ-pyridylmethylamine was heated for 17 hours at about 150° as described above for the benzyl analog. The reaction mixture was taken up in acetone and cooled to yield a precipitate which was filtered, washed with cold acetone, and dried *in vacuo* over phosphorus pentoxide to yield 118 mg. of somewhat hygroscopic material, m.p. 227–230° dec.

Anal. Calcd. for $C_{11}H_{11}N_7$: C, 54.76; H, 4.60; N, 40.65. Found: C, 55.11; H, 4.43; N, 40.02.

2-Amino-6-(2-phenylethyl)-aminopurine.—Following the same procedure previously described for the benzyl analog, 500 mg. of 6-methylthioguanine and 3 ml. of phenylethylamine were heated for 18 hours at about 150°. The reaction mixture was taken up in ether, and reduced to a viscous oily mass *in vacuo*. This residue was repeatedly extracted with hot water and the clear supernatants were combined, and cooled

(11) C. G. Skinner, J. R. Claybrook, F. D. Talbert and W. Shive, *Arch. Biochem. Biophys.*, **65**, 567 (1956).

(12) The ultraviolet absorption spectra were determined on a Beckman model DK-2 recording spectrophotometer, and the elemental analyses were carried out in the authors' laboratories by Mr. W. H. Orme-Johnson and Miss Judith Morehead. The 6-thioguanine was purchased from Francis Earle Biochemicals, Peekskill, N. Y., and the gibberellin was a commercial sample produced by Merck and Co. and labeled "Gibrel."

(13) J. A. Montgomery and L. B. Holum, THIS JOURNAL, **79**, 2188 (1957), reported a m.p. of 239–240° for this compound using dimethyl sulfate as the methylating agent.

in a refrigerator overnight to yield 75 mg. of needles, m.p. 202–203° dec.

Anal. Calcd. for $C_{13}H_{14}N_6$: C, 61.44; H, 5.55. Found: C, 61.43; H, 5.38.

Biological Assays.—The microbiological assay procedure with *Lactobacillus arabinosus* 17–5 has been previously described in detail.[6] The general technique for the lettuce seed germination study has been reported[4,14]; however, in the present study, the pre-soaked seeds were placed on filter paper wet with water alone for germination at 30° in the dark.

(14) C. G. Skinner, F. D. Talbert and W. Shive, *Plant Physiol.*, **33**, 190 (1958).

AUSTIN, TEXAS

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1] AND THE VETERINARY MEDICAL RESEARCH INSTITUTE, IOWA STATE COLLEGE[2]]

Possible Toxic Factor of Trichloroethylene-extracted Soybean Oil Meal[3]

BY L. L. MCKINNEY, J. C. PICKEN, JR.,[4] F. B. WEAKLEY, A. C. ELDRIDGE, R. E. CAMPBELL, J. C. COWAN AND H. E. BIESTER[4]

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Treatment of amino acids and peptides with trichloroethylene, under conditions known to produce the bovine aplastic anemia-causing factor in soybean oil meal and other proteins, showed that trichloroethylene reacted readily with the sulfhydryl groups of cysteine and reduced glutathione. The S-dichlorovinyl derivatives of L-cysteine and L-glutathione were synthesized and found to produce the aplastic anemia syndrome in calves typical of that produced by trichloroethylene-extracted soybean oil meal. The stability and properties of the S-dichlorovinyl derivatives are in agreement with known properties and mode of formation of the toxic principle in the meals. Enzymic hydrolysis of a toxic protein from trichloroethylene-extracted soybean oil meal resulted in the loss of a large portion of its toxicity. Chromatographic studies on the hydrolyzate indicated the presence of products resembling the synthetic compounds, and giving tests for the S-dichlorovinyl group.

Earlier studies in these laboratories[5] have shown that the bovine aplastic anemia-causing factor in trichloroethylene-extracted soybean oil meal (TESOM) is associated with the purified protein component of the meal, that it is labile to vigorous acid hydrolysis and that measurable loss in toxicity occurred on heating the alkaline (*p*H 11–12) protein dispersion at 60° for 4 hours. Independent studies[6] confirmed these findings and showed that a suspension of the meal in dilute sulfuric acid at *p*H 1.5 at 65–70° for 48 hr. retained its toxicity. Picken and Biester[7] have shown that the aplastic anemia-causing factor is formed by heating zein, casein, lactalbumin and gelatin, as well as soybean protein with trichloroethylene (TCE) at 120°, and that the presence of lysine, tryptophan and tyrosine in the protein does not appear essential for the formation of the toxic entity.

A preliminary report[8] listed the findings that prompted the synthesis of S-(dichlorovinyl)-L-cysteine which on oral administration to calves produced an aplastic anemia syndrome typical of that produced by TESOM. The present paper describes the details of these experiments.

(1) One of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois. Article not copyrighted.

(2) A report of work done, in part, under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act. Contract supervised by the Northern Utilization Research and Development Division.

(3) Presented before the Division of Biological Chemistry, 132nd Meeting, American Chemical Society, New York, N. Y., Sept. 8–13, 1957.

(4) Veterinary Medical Research Institute, Iowa State College, Ames, Iowa.

(5) L. L. McKinney, F. B. Weakley, R. E. Campbell, A. C. Eldridge, J. C. Cowan, J. C. Picken, Jr., and N. L. Jacobson, *J. Am. Oil Chemists' Soc.*, **34**, 461 (1957).

(6) T. A. Seto, M. O. Schultze, V. Perman, F. W. Bates and J. H. Sautter, *J. Agr. Food Chem.*, **6**, 49 (1958).

(7) J. C. Picken, Jr., and H. E. Biester, Abst. Papers, 132nd Meeting, Am. Chem. Soc., New York, N. Y., Sept. 8–13, 1957.

(8) L. L. McKinney, F. B. Weakley, A. C. Eldridge, R. E. Campbell, J. C. Cowan, J. C. Picken, Jr., and H. E. Biester, THIS JOURNAL, **79**, 3932 (1957).

Results and Discussion

Sealed Tube Experiments.—Sealed tube interaction of amino acids with TCE at 120° for 12 hr. slightly decreased the ninhydrin color of DL-asparagine, L-glutamic acid and DL-lysine hydrochloride (Table I) as determined by chromatogram assay. Interaction of cysteine with TCE in sealed tubes liberated hydrogen sulfide together with a significant amount of chloride; the ninhydrin values were inconsistent and are not included in Table I. The presence of steel wool in the cysteine–TCE reaction prevented the liberation of hydrogen sulfide and resulted in the formation of an additional ninhydrin-positive spot observed by paper chromatography.[9] This new ninhydrin-positive spot absorbed ultraviolet light and gave a color reaction with 4-(*p*-nitrobenzyl)-pyridine (4-NBP).[10]

Sealed tube experiments indicated that TCE reacted more readily with glutathione than it did with cysteine. Paper chromatograms of the glutathione–TCE reaction mixture and admixtures revealed a ninhydrin-positive spot which absorbed ultraviolet light and gave a 4-NBP test identical to that given by the compound S-(dichlorovinyl)-L-cysteine. Occasionally two spots, typical of the S-dichlorovinyl derivatives of cysteine and glutathione, appeared on paper chromatograms of glutathione–TCE reaction mixtures. Usually, only the spot associated with S-(dichlorovinyl)-L-cysteine was in evidence, indicating a breakdown of the peptide in the reaction mixture at 120° over the 20-hour period.

Table II records the relative amounts of inorganic chloride liberated by sealed tube interaction of various amino acids and peptides with TCE at approximately 120°. No increase in chloride

(9) M. Gutcho and L. Laufer, "Paper Chromatography of Glutathione and Its Hydrolysis Products," in "Glutathione, A Symposium," Academic Press, New York, N. Y., 1954, pp. 79–87.

(10) (a) T. A. Geissman, H. Hochman and R. T. Fukuto, THIS JOURNAL, **74**, 3313 (1952); (b) J. Epstein, R. W. Rosenthal and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955).