508. *Purines, Pyrimidines, and Imidazoles. Part XX.*[1] *Some Syntheses of 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxyamide 5′-Phosphate.*

By G. Shaw, D. V. Wilson, and (in part) C. P. Green.

Three syntheses of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxyamide 5′-phosphate (AICAR) from the isopropylidene methyl ester (II) are described. They involve (*a*) phosphorylation of (II) with mono-2-cyanoethyl phosphate and dicyclohexylcarbodi-imide, or with pyrophosphoryl chloride, followed by amidation of the ester group; (*b*) amidation of the ester group then phosphorylation of the amide with mono-2-cyanoethyl phosphate and dicyclohexylcarbodi-imide, and (*c*) hydrolysis of (II) with alkali, reaction of the resulting carboxylic acid with ammonia and dicyclohexylcarbodi-imide, then phosphorylation as in (*b*).

The phosphate (I) is a central intermediate in the biosynthesis *de novo* of purine nucleotides (see ref. 2 for reviews). We have been interested in obtaining relatively large quantities of the pure compound, preferably by a method which could also be readily

[1] Part XIX, Shaw and Wilson, *J.*, 1963, 1077.
[2] Buchanan and Hartman, *Adv. Enzymol.*, 1959, **21**, 199; Hartman and Buchanan, ***Ann. Rev. Biochem.***, 1959, **28**, 365.

adapted to the preparation of analogous nucleotides which in turn might act as competitive inhibitors of the enzyme system concerned with the cyclisation of (I) into inosinic acid.

Two preparations of the phosphate (I) have been described recently. The first [3] is a variation of an earlier route [4] to the corresponding riboside, and involves the methoxymethylation of 2′,3′-isopropylideneinosine 5′-di-*p*-nitrophenyl phosphate with sodium hydride and chloromethyl methyl ether, and subsequent ring-opening of the product with sodium hydroxide, followed by enzymic removal of the *p*-nitrophenyl groups with the venom of *Crotalus adamanteus*. A yield of 11—16% is claimed. However, no information is given in the Paper about the purity of the final product, and in addition it is pointed out somewhat discouragingly, both in the Discussion and Experimental sections of the Paper, that after the alkaline hydrolysis, unless the optical density ratio of the material at 270/250 mμ is about 1·3 " completion of a given preparation was not worthwhile " or " proceeding with the preparation may be inadvisable." The second preparation [5] of compound (I) involves the reaction of inosinic acid with 0·5N-hydrochloric acid, zinc dust, and aqueous ammonium chloride for a few minutes. Unfortunately this promising method gives also a second arylamine which has a u.v. spectrum identical with that of the phosphate (I); also it does not separate from (I) at all readily on ion-exchange columns.

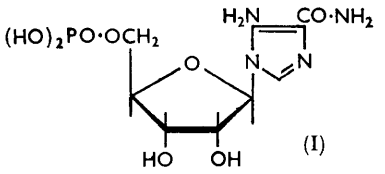
Since both these methods give rise to material of doubtful purity and in variable and uncertain yield we have turned our attention to an examination of alternative, more reliable preparative methods.

A central compound in our experiments has been the isopropylidene-ester (II) which can readily be obtained crystalline in high yield by reaction of the corresponding unprotected nucleoside with acetone in the presence of toluene-*p*-sulphonic acid as reported by us in an earlier publication.[6] The compound may also be prepared by reaction of the nucleoside with acetone, 2,2-dimethoxypropane, and Dowex 50 H^+ resin as an acid catalyst. This last method has the advantage of speed in working up but gives a somewhat lower yield. We now describe three methods for the conversion of the isopropylidene ester (II) into AICAR (I).

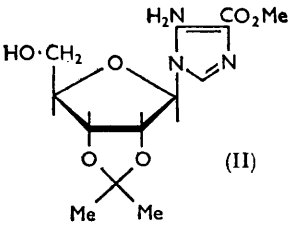
(i) Phosphorylation of the ester (II) with diphenylphosphorochloridate and attempted reduction of the crude product gave, after mild acid treatment, only the diphenyl phosphate ester (III; R = Ph). However, phosphorylation of (II) with either mono-2-cyanoethyl phosphate [7] and dicyclohexylcarbodi-imide or preferably with pyrophosphoryl chloride gave, after acid treatment to remove the isopropylidene group, the phosphorylated ester (III; R = H) which was readily purified by ion-exchange chromatography, and obtained analytically pure in good yield. Subsequent reaction of this nucleotide with aqueous ammonia at 100° in a sealed tube then gave the required AICAR (I) in 40—50% yield. Paper chromatography of the crude product showed it to be free from the corresponding aglycone or nucleoside, and it was readily obtained pure by ion-exchange chromatography. The identity of the synthetic AICAR was confirmed, in the absence of an authentic specimen, by microanalysis, by ultraviolet spectroscopy at various pH values, when values very similar to those quoted [8] for the natural material were obtained, by the absorption spectra of the dyestuff produced in the Bratton–Marshall test [9] for arylamines, by conversion into inosinic acid with formic–acetic anhydride [8] and identification of this acid by comparison on paper chromatograms with an authentic specimen, and finally by the enzymic conversion of the phosphate into 5-amino-1-β-D-ribofuranosylimidazole-4-carbonyl-L-aspartic

[3] Shaw, *J. Amer. Chem. Soc.*, 1961, **83**, 4770.
[4] Shaw, *J. Amer. Chem. Soc.*, 1958, **80**, 3899; 1959, **81**, 6021.
[5] Private communication from Dr. M. Sevag, also mentioned in Miller and Buchanan, *J. Biol. Chem.*, 1961, **237**, 485.
[6] Shaw and Wilson, *J.*, 1962, 2937.
[7] Tener, *J. Amer. Chem. Soc.*, 1961, **83**, 159.
[8] Flaks, Erwin, and Buchanan, *J. Biol. Chem.*, 1957, **228**, 201.
[9] Bratton and Marshall, *J. Biol. Chem.*, 1939, **128**, 537.

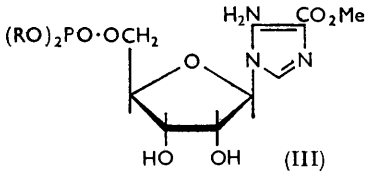
acid 5′-phosphate (SAICAR) in the presence of fumaric acid and a preparation of Adenylosuccinate AMP-lyase from yeast in a phosphate buffer.[10] The properties of the compound formed in this last reaction in almost quantitative yield, were in excellent agreement with



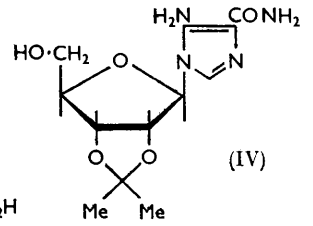
(I)



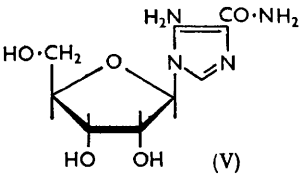
(II)



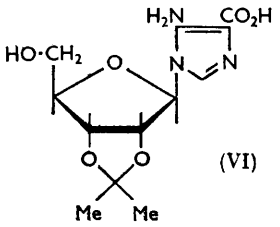
(III)



(IV)



(V)



(VI)

those of the natural SAICAR prepared earlier by us.[1] The AICAR prepared by this method was used as a standard and other preparations were compared directly with it.

(ii) Reaction of the ester (II) with aqueous ammonia in a sealed tube at 100° gave a compound identical on paper chromatograms in various solvents with the substance, presumably the carboxyamide (IV), which was also obtained from compound (V) with acetone and di-*p*-nitrophenyl phosphate, but not crystallised. However, the structure of this intermediate was confirmed by mild acid hydrolysis to (V) which was isolated as a crystalline picrate having the same melting point and infrared spectrum as the picrate of an authentic specimen of the natural base. Subsequent phosphorylation of the carboxyamide (IV) with mono-2-cyanoethyl phosphate and dicyclohexylcarbodi-imide, and removal of the isopropylidene group then gave AICAR (I), but in low yield.

(iii) Hydrolysis of the ester (II) with aqueous sodium hydroxide gave, presumably, the acid (VI), which with ammonia and dicyclohexylcarbodi-imide in anhydrous pyridine, followed by phosphorylation with mono-2-cyanoethyl phosphate and dicyclohexylcarbodi-imide and removal of the isopropylidene group gave a small yield of AICAR. The low yield in this reaction was somewhat surprising since better yields have been obtained [1] in the analogous, and superficially at least less favourable synthesis of the *N*-succino-derivative (SAICAR) by acylation of L-aspartic acid esters.

The first of these methods proved to be much the best of the three and is clearly capable of extension to allow for the preparation of substituted carboxyamides by using primary or secondary amines in place of ammonia.

Experimental

Unless otherwise stated, evaporations were carried out in a Buchi rotary evaporator, under water-pump vacuum, with a flask temperature of 40° or less.

[10] Miller, Lukens, and Buchanan, *J. Biol. Chem.*, 1959, **234**, 1806.

Paper chromatograms were run on unwashed Whatman No. 1 paper in the systems (A) n-butanol–acetic acid–water (12 : 3 : 5) ascending; (B) isobutyric acid–ammonia solution (*d* 0·88)–water (66 : 1 : 33) descending; (C) n-propanol–0·2N-ammonium hydroxide (3 : 1) ascending, and (D) n-butanol saturated with water, ammonia atmosphere in the tank, ascending. Papers were dried in air and spots detected by examination under a u.v. lamp, by the modified Bratton–Marshall spray reagents [11] or the ammonium molybdate reagent of Burrows.[12] Barium or lithium salts were treated with a slight excess of Zeo-Karb 225 H^+ resin before chromatography.

Ion-exchange separations were performed in an apparatus, all Teflon or glass, equipped with a Buchler micropump for accurate control of flow rates, and an LKB Uvicord 4701A ultraviolet absorptiometer with a flow cell of 3-mm. light path for continuous recording of the column eluates at 253·7 mμ. All resins used for ion-exchange chromatography were an analytical grade of Dowex prepared and marketed by Bio-Rad Laboratories, Richmond, California.

U.v. spectra were measured in a Perkin-Elmer 137UV recording spectrophotometer, and i.r. spectra on a Perkin-Elmer 237 spectrophotometer, the potassium bromide disc technique being used.

A standardised procedure was used for quantitative measurements involving the Bratton–Marshall test.

Methyl 5-Amino-1-(2,3-O-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate.—AG 50W × 8 (100—200 mesh) resin (H^+ form) was stored overnight under dry methanol containing about 10% of 2,2-dimethoxypropane, and then washed successively with dry methanol and dry acetone. This resin (2·5 ml.) and 2,2-dimethoxypropane (6 ml.) were added to a suspension of methyl 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylate [6] (0·2 g.) in dry acetone (60 ml.), and the mixture shaken vigorously at room temperature in a stoppered flask for 3 hr.; the nucleoside dissolved in 45 min. The dark red resin was filtered off, rapidly washed with dry acetone (10 ml.), and immediately shaken with N-ammonia solution (30 ml.). The extraction with ammonium hydroxide was repeated three times. The pale yellow extracts were combined and evaporated to a solid which was extracted under reflux with ethyl acetate (100 ml.). Partial evaporation of the ethyl acetate extract gave prisms (112 mg.) of methyl 5-amino-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate, identical [m. p., mixed m. p., i.r. spectrum, and paper chromatography in system (A)] with samples prepared previously.[6]

Methyl 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxylate 5′-(Diphenyl Phosphate).—A solution of the foregoing isopropylidene-ester (0·102 g.) in anhydrous pyridine (6 ml.) was cooled to 0° and slowly treated with a cooled solution of diphenyl phosphorochloridate (0·225 g.) in pyridine (2·5 ml.). The solution was allowed to warm to room temperature, then set aside for 24 hr. Water (1 ml.) was added and the solution evaporated to dryness. The resulting gum was evaporated several times with small quantities of water, then washed by decantation with water, and finally dried by evaporation with ethanol (2 × 5 ml.) then benzene (5 ml.). The gum was dissolved in acetic acid (20 ml.) and exposed to hydrogen and platinic oxide (20 mg.) but there was only a very slight absorption of hydrogen. The solution was evaporated to small volume then heated, after addition of water, for 1·5 hr. at 100°. The solution was evaporated to dryness, the residue was dissolved in aqueous ammonia, and the pH of the solution adjusted to 7·8. This solution was passed through a column of AG1 × 2 (200—400 mesh) resin (Br^- form), and fractions were eluted with 0·01N-hydrobromic acid. The fraction between 90 and 310 ml. was collected and adjusted to pH 7. Evaporation of the solution to about half-volume gave the *nucleotide ester* (19 mg.) as needles, m. p. 70—73° (Found: C, 52·05; H, 5·05; N, 9·5. $C_{22}H_{24}N_3O_9P$ requires C, 52·3; H, 4·8; N, 8·3%); the nitrogen analysis was carried out on only 1·364 mg. of material which may explain the poor value.

Methyl 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxylate 5′-Phosphate.—(*a*) A solution of the foregoing isopropylidene ester (0·47 g.) in dry pyridine (30 ml.) was treated with 2-cyanoethyl phosphate in pyridine [7] (8 ml. containing 8 mmoles) and evaporated to a clear gum. Anhydrous pyridine (10 ml.) was added, the solution was evaporated, and the residue dissolved in anhydrous pyridine (20 ml.). Dicyclohexylcarbodi-imide (4 g.) was added and the mixture

[11] Baddiley, Buchanan, Hardy, and Stewart, *J.*, 1959, 2893.
[12] Burrows, Grylls, and Harrison, *Nature*, 1952, **170**, 800.

set aside for 3 days at room temperature in a closed flask. After addition of water (10 ml.), the mixture was evaporated to dryness, and the residue was again evaporated with water (25 ml.). The pyridine-free gum was heated at 100° for 1·75 hr. in 15% acetic acid, the solution evaporated to dryness, and the residue freed from acetic acid by repeated evaporation with water. The product was heated at 100° for 30 min. in an open flask with ammonia solution (5 ml.; *d* 0·88) and water (25 ml.). The dark brown suspension was filtered, and the collected precipitate of dicyclohexylurea was washed with dilute ammonia solution (100 ml.). The filtrate and washings were combined, evaporated to 80 ml., and adjusted to pH 9 with ammonia solution. This solution was placed on a column (2 cm. × 9 cm. of AG 1 × 2 (200—400 mesh) resin (Br^- form), and the column washed with water (350 ml.). Elution commenced with 0·008N-hydrobromic acid at a flow rate of 60 ml./hr. Strongly absorbing (at 253·7 mμ) material appeared between 270 and 410 ml. (fraction *A*) and between 720 and 1050 ml. (fraction *B*). Fraction (*A*) gave a negligible colour in the Bratton–Marshall test and was discarded. Fraction *B* gave a strong purple colour, and quantitative measurements indicated about 127 mg. of the desired compound to be present. This fraction was adjusted to pH 8 with saturated barium hydroxide solution and evaporated to 30 ml. The flocculent precipitate which separated was removed by centrifugation, washed with water (3 ml.), and discarded. The combined supernatant solution and washings, with ethanol (120 ml.) and M-barium bromide solution (1 ml.), gave a precipitate of *methyl 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylate-(barium 5′-phosphate)* (157 mg.) which was collected after 30 min. at 0°, by centrifugation, washed with absolute ethanol (3 × 5 ml.) and dry ether (5 ml.), and dried *in vacuo* over solid potassium hydroxide and calcium chloride. The material was identical with that prepared in (*b*), for which analytical data were obtained.

(*b*) The foregoing isopropylidene ester (0·104 g.) was added to redistilled pyrophosphoryl chloride (0·25 ml.) at −25°, and the mixture stirred whilst the temperature was allowed to rise to that of the room. The mixture was set aside at room temperature for 1·5 hr., cooled to −20°, and treated with barium acetate (1·2 g.) in water (3 ml.). The solution was diluted with water (20 ml.), the pH adjusted to 6·5 with saturated barium hydroxide solution, and the solution boiled for a few minutes and filtered, and the precipitate washed with hot water (2 × 5 ml.). The combined solution and washings were evaporated to about 10 ml., heated for 1·5 hr. on a water-bath, after the addition of acetic acid (1 ml.)., and evaporated with water until free from acetic acid. The residue was dissolved in water and the pH adjusted to 7·6 with dilute ammonium hydroxide solution. The mixture was chromatographed as under (*a*) to give the *barium salt monohydrate* (101 mg.) (Found: C, 23·5; H, 3·35; N, 8·15; P, 6·35; Ba, 26·8. $C_{10}H_{14}BaN_3O_9,H_2O$ requires C, 23·7; H, 3·2; N, 8·3; P, 6·1; Ba, 27·1%); $\lambda_{max.}$ (in water) 271 mμ (ε 13,460); $\lambda_{max.}$ (in 0·1N-hydrochloric acid) 268 mμ (ε 12,180), λ_{sh} 245—253 mμ (ε 9,610); $\lambda_{max.}$ (at pH 9), 271 mμ (ε 13,940); in the Bratton–Marshall test the nucleotide gave a strong purple colour ($\lambda_{max.}$ 541 mμ). In solvent system (A) it had R_F 0·26.

Action of Ammonium Hydroxide on Methyl 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxylate 5′-Phosphate. Preliminary Experiments.—(*a*) The ammonium salt of the methyl ester in ammonia solution (*d* 0·88) at a concentration of *ca.* 1 mg./ml. was heated in a sealed tube at 100° for 4 hr. Examination of the mixture by paper chromatography (system A) showed the presence of unchanged ester, plus material later identified as AICAR, as the only products which gave a colour with the Bratton–Marshall spray reagents and which absorbed ultraviolet light. Under similar conditions the corresponding methyl ester riboside was partly converted into the nucleoside corresponding to AICAR, plus a trace of material tentatively identified as the ester aglycone. Under the same conditions the mixture from an authentic sample of the nucleoside corresponding to AICAR, gave only a single spot of starting material.

(*b*) At a concentration of 4 mg./ml. in ammonia solution (*d* 0·88) in a sealed tube at 100° the methyl ester ribotide gave, after 8 hr., AICAR as the major product, with a trace of aglycone and some unchanged material. In each experiment no ester riboside or riboside of AICAR was formed.

Preparation of 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxyamide 5′-Phosphate (AICAR). —(*a*) A solution of methyl 5-amino-1-β-D-ribofuranosylimidazole-4-carboxylate 5′-phosphate (23 mg.) prepared from the foregoing barium salt using Zeo-Karb 225 resin (H^+ form), in ammonium hydroxide (6 ml.; *d* 0·88) was divided between two tubes (1·5 cm. × 10 cm.) which were sealed and heated for 8 hr. at 100°. The resulting pale yellow solutions contained a trace of a colourless crystalline precipitate which dissolved when the suspensions were combined

and evaporated to *ca.* 1 ml. This solution was diluted to 50 ml. with water, adjusted to pH 9·5 with aqueous ammonia, placed on a column of AG 1 × 8 (200—400 mesh) resin in the Cl^- form, and eluted with 0·005N-hydrochloric acid at a flow rate of 45 ml./hr. Ultraviolet-absorbing material appeared between 70 and 90 ml. (fraction *A*), 100 and 160 ml. (fraction *B*), and 190 and 300 ml. (fraction *C*). Fraction (*A*) gave a negligible colour with the Bratton–Marshall reagents and was discarded. Fraction (*B*) (~3 mg.) had $\lambda_{max.}$ 268—270 mμ in acid and $\lambda_{max.}$ 272 mμ in alkali, gave a purple colour ($\lambda_{max.}$ 535 mμ) in the Bratton–Marshall test, and was identical on paper chromatograms with the starting material. Fraction (*C*) gave a single ultraviolet-absorbing spot, also positive in the Bratton–Marshall test (R_F 0·12 in system A, 0·06 in system C) and was identified as AICAR (yield 10 mg., or 52%) by ultraviolet spectroscopy (broad shoulder 247 mμ, $\lambda_{max.}$ 268—269 mμ in acid, $\lambda_{max.}$ 269 mμ in alkali), by formation of a purple colour ($\lambda_{max.}$ 539—540 mμ) in the Bratton–Marshall test, and by enzymic conversion into 5-amino-1-β-D-ribofuranosylimidazole-4-carbonyl-L-aspartic acid 5′-phosphate (SAICAR) as follows. A portion (3·5 ml.) of fraction (*C*), 0·2M-potassium dihydrogen phosphate solution (2 ml.), and fumaric aid (40 mg.) were mixed, the solution was adjusted to pH 7·2 with dilute potassium hydroxide solution, and diluted to 10 ml. with water. Portions (2 ml.) were incubated at 37° for 10 min. with a solution containing Adenylosuccinate AMP-lyase (0·2 ml.) prepared from yeast [13] provided by the United Yeast Company, Brighouse, Yorkshire, and shown in preliminary experiments to be free from SAICAR, and AICAR. The reaction was terminated by addition of 10% trichloroacetic acid (1 ml.), protein was removed by centrifugation, and the supernatant solution examined by the Bratton–Marshall test, with appropriate blanks and controls. The purple solution produced from 1·5 ml. of supernatant solution by the standard procedure at room temperature to give a final volume of 5 ml., had $\lambda_{max.}$ 550 mμ (optical density 0·06) and, when the test was carried out a 0°, $\lambda_{max.}$ 555—560 mμ (optical density 0·29) corresponding to almost quantitative conversion into SAICAR of the material of fraction (*C*). In addition, the optical-density ratio at 540 : 600 mμ of this solution prepared at 0° was 1·29, in excellent agreement with the reported values for SAICAR.[1]

In a similar experiment, the methyl ester ribotide (0·07 g.) gave, after chromatography, a solution of AICAR which was neutralised to pH 8 with aqueous barium hydroxide solution. The *barium salt* of AICAR was precipitated by addition of ethanol (4 vol.) to the concentrated and clarified aqueous solution, purified by precipitation from an aqueous solution with ethanol, and finally washed with ethanol and ether and dried *in vacuo* at room temperature; it retained some ethanol (Found: C, 24·4; H, 3·8; N, 10·3; P, 5·6; Ba, 24·9. $C_9H_{12}BaN_4O_8P,C_2H_6O,1\frac{1}{2}H_2O$ requires C, 24·2; H, 3·9; N, 10·3; P, 5·7; Ba, 25·15%).

The identity of this substance was further confirmed by formylation and cyclisation to inosine-5′ phosphate. A mixture of acetic anhydride (0·25 ml.) and 98—100% formic acid (0·25 ml.) was added to a solution of the lithium salt of AICAR (1·5 mg.) in 98—100% formic acid (0·5 ml.). After 100 min. at 37°, the solution was evaporated *in vacuo* at room temperature to a solid which was heated for 70 min. at 37° in 0·1N-sodium hydroxide solution, cooled, treated with Zeo-Karb 225 resin (H^+ form) to remove cations, and examined by paper chromatography. The major ultraviolet-absorbing spot, which did not give a colour with the Bratton–Marshall spray reagents, was identified as inosine-5′ phosphate by comparison (systems A and B) with an authentic sample. A small amount of unchanged AICAR was also present in the mixture, and separated best from inosine-5′ phosphate in system (B).

(*b*) (i) A solution of methyl 5-amino-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate (50 mg.) in ammonia solution (*d* 0·88; 5 ml.) was heated in a sealed tube (11 cm. × 1·5 cm.) for 15 hr. at 100°, and the clear solution was evaporated to an almost colourless glass. Paper chromatography and development by the Bratton–Marshall spray reagents revealed a single product (yield 65%, estimated spectroscopically) as a purple spot [R_F 0·8 in system (A) and 0·85 in system (C)]. No trace of starting material, aglycones, or free nucleosides could be detected.

The product, which did not crystallise, was heated at 100° for 1·75 hr in 10% aqueous acetic acid and then evaporated to a gum which was identical on paper chromatograms (systems A and C) with an authentic specimen of the nucleoside corresponding to AICAR and purchased from the California Corporation for Biochemical Research, Los Angeles, U.S.A. Addition of saturated aqueous picric acid to a solution of the synthetic material in water gave bright yellow

[13] Carter and Cohen, *J. Biol. Chem.*, 1956, **222**, 17.

needles identical (m. p., mixed m. p., and infrared spectrum) with the picrate [14] prepared similarly from the authentic sample. (ii) 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxyamide (0·1 g.), prepared by the method of Shaw *et al.*[14] and purified by chromatography on AG 50W × 8 resin, was suspended in dry acetone (10 ml.) containing 2,2-dimethoxypropane (1 ml.). Di-*p*-nitrophenyl phosphate [15] (0·3 g.) was added, and the mixture was stirred at room temperature with exclusion of moisture. All solids dissolved within 1 hr. After a further 5 hr. the yellow solution was added dropwise with vigorous stirring to ice-cold 0·5N-ammonium hydroxide solution (40 ml.). Paper chromatography of the solid residue obtained by evaporation showed absence of starting material and presence of a major product which gave a purple colour with the Bratton–Marshall spray reagents and was identical with the first material described under (i) above. Traces of a faster-running diazotisable product were also detected. The residue was dried by evaporation with ethanol (2 × 15 ml.) and dry pyridine (2 × 5 ml.) then treated with the mono-2-cyanoethyl phosphate–pyridine reagent (1 ml. containing 1 mmole), and evaporated to a gum. This was thrice evaporated with dry pyridine, and finally dissolved in dry pyridine (10 ml.). Dicyclohexylcarbodi-imide (0·99 g.) was added and the clear solution set aside at room temperature with exclusion of moisture for 40 hr., then treated with water (5 ml.) and, after a further 2 hr. at room temperature, evaporated to dryness. The resulting gum was twice evaporated with water to remove pyridine, heated at 100° during 90 min. with 10% aqueous acetic acid (20 ml.), evaporated to dryness, freed from acetic acid by repeated evaporation with water, and heated at 60° for 90 min. with ammonia solution (*d* 0·88; 20 ml.) and water (10 ml.). The mixture was filtered and the collected dicyclohexylurea washed with water until the washings failed to give a colour with the Bratton–Marshall reagents. The filtrate and washings were combined and evaporated to a gum, which was extracted with small portions of water (total 10 ml.), and insoluble material discarded. The extracts were combined and adjusted with saturated barium hydroxide solution to pH 8·5, treated with M-barium bromide solution (2 ml.), and set aside at room temperature for 1 hr. The precipitate was separated by centrifugation, washed with water, and discarded. Addition of ethanol (60 ml.) to the combined supernatant liquid and washings gave a flocculent precipitate which, after 30 min. at 0°, was collected by centrifugation and washed with 90% ethanol (10 ml.), ethanol (2 × 10 ml.), and dry ether (10 ml.), and dried *in vacuo* over phosphoric oxide at room temperature. This crude barium salt contained AICAR (11 mg., *ca.* 8% yield from the riboside, estimated spectroscopically) paper-chromatographically identical (systems A and C) with the sample described above. It gave a purple colour ($\lambda_{max.}$ 540 mμ) in the Bratton–Marshall test, and was completely free of riboside or aglycone.

(*c*) Methyl 5-amino-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate (0·257 g.) was hydrolysed to the acid which was converted into the pyridine salt as previously described.[1] This salt, in dry pyridine (10 ml.), was treated at 0° with a large excess of ammonia in dry ether, dicyclohexylcarbodi-imide (0·5 g.) was added, and the resulting emulsion was set aside at room temperature for 68 hr. in a closed flask. The mixture was evaporated to 15 ml., diluted with dry pyridine (10 ml.), and again evaporated to 15 ml. The mono-2-cyanoethyl phosphate–pyridine reagent [7] (4 ml. containing 4 mmoles) and dicyclohexylcarbodi-imide (3·5 g.) were added, and the gel-like solution was kept at room temperature for 39 hr. with exclusion of moisture. After addition of water (10 ml.) and storage at room temperature for a further 1·5 hr., the suspension was evaporated to dryness, freed from pyridine by evaporation with water, heated for 2 hr. at 100° with dilute acetic acid, and again evaporated. The residue was repeatedly evaporated with water then kept at 55—65° for 1·5 hr. in a mixture of ammonia solution (*d* 0·88; 40 ml.) and water (20 ml.); some darkening occurred during this treatment. Evaporation of the solution gave a solid which was extracted several times with water (total 120 ml.), and the combined extracts were evaporated to *ca.* 30 ml., adjusted to pH 8·5 with saturated barium hydroxide solution, and treated with M-barium bromide solution to give a precipitate which was separated by centrifugation, washed twice with water, and discarded, The combined aqueous solutions were mixed with ethanol (4 vol.) to give the barium salt of AICAR which was collected by centrifugation and washed with 90% ethanol (10 ml.), ethanol (2 × 10 ml.), and dry ether (10 ml.).

This sample contained 22 mg. of AICAR (estimated spectroscopically, *ca.* 8% yield) free

[14] Shaw, Warrener, Butler, and Ralph, *J.*, 1959, 1648.
[15] Hampton, *J. Amer. Chem. Soc.*, 1961, **83**, 3640.

from ultraviolet-absorbing impurities, and was chromatographically identical (systems A and C) with the sample described above under (i). It gave a purple colour ($\lambda_{max.}$ 540 mμ) in the Bratton–Marshall test.

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