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Investigations on Pantothenic Acid and Its Related Compounds. XXIII.[1] Chemical Studies. (10).[2] Chemical Synthesis of Coenzyme A Analogs of a Modified Purine Base Moiety[3]

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Two coenzyme A analogs having purine base different from adenine, such as inosino- (IV) and guano-coenzyme A (IX), have been synthesized by application of the method *via* the thiazoline intermediate in the same manner as the total synthesis of coenzyme A. Inosino-coenzyme A has been also obtained by deamination with nitrite from the disulfide form of coenzyme A. Synthesis of guano-coenzyme A has been more efficiently effected by a combination of the methods of Moffatt and Khorana, for the formation of pyrophosphate, and of Michelson, for 2′,3′-cyclic phosphate fission, than the thiazoline method.

Viewed from the chemical structure, many coenzymes of nucleotide type occure in the form of adenosine derivatives. It seems interesting and desirable to synthesize the unnatural compounds modified in the nucleoside moiety and examine their effects on the enzymic reactions concerned. No work on the coenzyme A (CoA) analog along this line has been known.[5] Following the previous work on CoA analogs of modified cysteamine moiety,[6] further step was taken in order to bridge the above gap. The present report deals with the syntheses of inosino- and guano-CoA.

The adenine moiety of nucleoside, nucleotide or nucleic acid is usually converted to the hypoxanthine moiety by deamination with nitrite in acidic medium.[7] Our attempt to apply this method to deamination of CoA (SH form) proved inefficient due to the concomitant oxidation of thiol group to the unknown substance. On the other hand, the thiazoline method developed by us[6,8,9] was successfully employed using the nitrile compound (II; inosino-CoA–CN) obtained from the key compound (I; CoA–CN) in the case of CoA synthesis. CoA–CN (I; P^1-adenosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate) was deaminated with nitrite to inosino-CoA–CN (II; P^1-inosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate) in 77% yield. According to the standard method, the trilithium salt of II was allowed to react with cysteamine until completion of thiazoline ring closure which was checked by disappearance of infrared (IR) absorption characteristic of the nitrile group. The thiazoline intermediate (III), without isolation, was hydrolyzed to the crude inosino-CoA

1) Part XXII: T. Suzuki, *Biochim. Biophys. Acta*, **191**, 559 (1969).
2) Part (9): O. Nagase, Y. Hosokawa, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **17**, 398 (1969).
3) A part of this work was preliminarily communicated in *Chem. Pharm. Bull.* (Tokyo), **14**, 683 (1966).
4) Location: *Minamifunabori-cho, Edogawa-ku, Tokyo, 132, Japan.*
5) Prof. Dr. C.J. Stewart, San Diago State College, San Diago, Cal., U.S.A., has synthesized cytidine-CoA according to his private letter of November 13, 1968 to M. Shimizu.
6) M. Shimizu, O. Nagase, Y. Hosokawa, and H. Tagawa, *Tetrahedron*, **24**, 5241 (1968).
7) N.O. Kaplan, "Methods in Enzymology," Vol. III, ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, N.Y., 1957, p. 873.
8) M. Shimizu, G. Ohta, O. Nagase, S. Okada, and Y. Hosokawa, *Chem. Pharm. Bull.* (Tokyo), **13**, 180 (1965).
9) M. Shimizu, O. Nagase, S. Okada, Y. Hosokawa, H. Tagawa, Y. Abiko, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **15**, 655 (1967).

(IV) by heating at acidic pH. Purification effected as in the cases of CoA[9] and other analogs[6] afforded the analytically pure inosino-CoA (IV; P^1-inosine 3′-phosphate 5′-P^2-D-pantetheine 4′-pyrophosphate) in 59.5% yield. The structure of IV was confirmed by paper chromatography of the acidic and the alkaline hydrolyzate showing a spot of inosine 3′,5′-diphosphate and of pantetheine 4′-phosphate, respectively. The former was identified with the sample synthesized by the deamination of adenosine 2′(3′),5′-diphosphate. Possesion of IV in the pure state led to reinvestigate deamination of CoA. When oxidized beforehand to the disulfide form with hydrogen peroxide, CoA was smoothly converted to inosino-CoA with nitrite in 64% yield. Chart 1 shows the above reaction sequences.

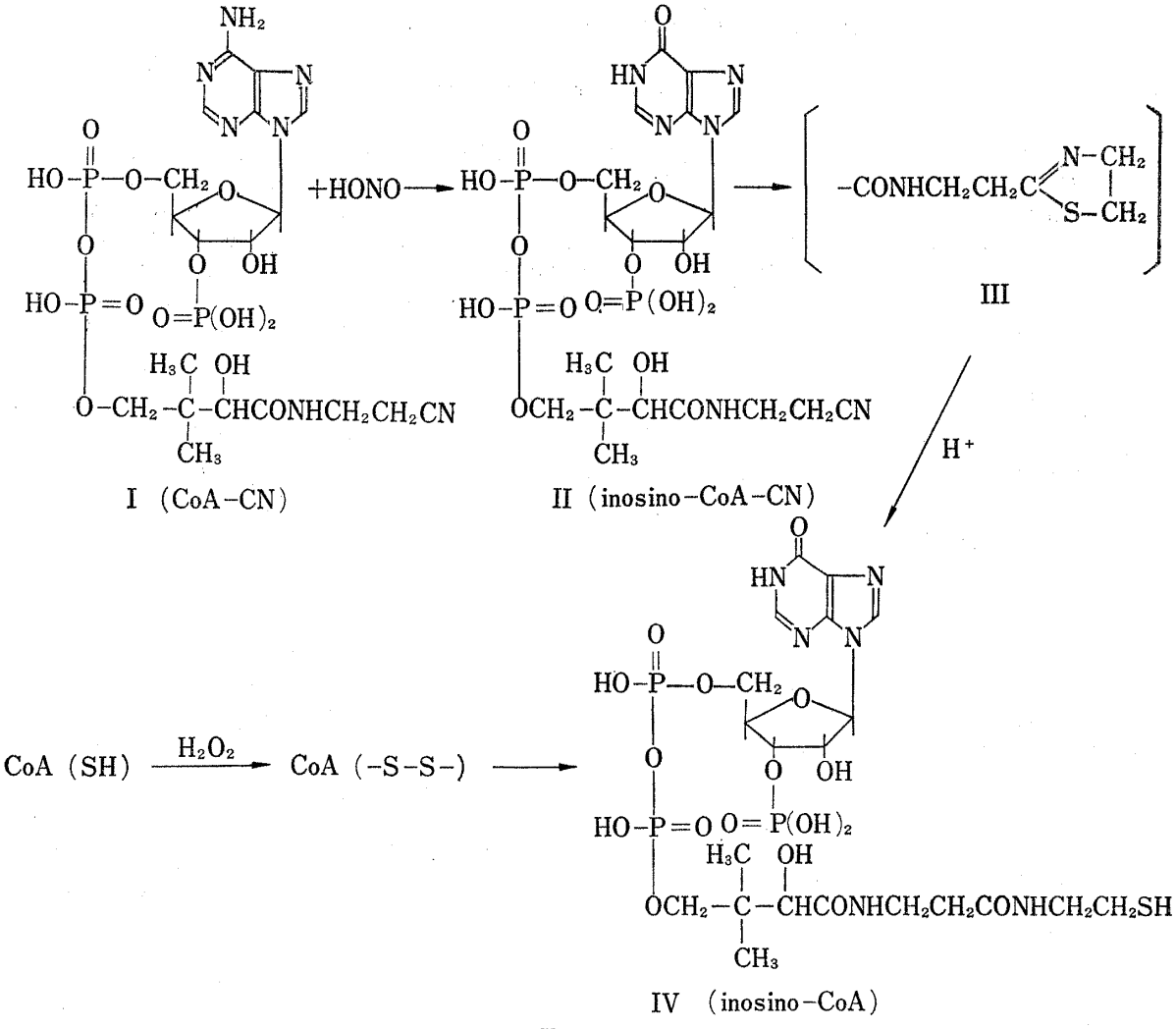

Chart 1

For the synthesis of guano-CoA (IX; P^1-guanosine 3′-phosphate 5′-P^2-D-pantetheine 4′-pyrophosphate), the thiazoline method was applied as shown in Chart 2. The yield, however, was considerably poor (7.2% from VII). The solubility of the guanosine derivatives in organic solvents is generally less than that of adenosine derivatives. The condensation reaction of guano-CoA–CN (VII; P^1-guanosine 3′-phosphate 5′-P^2-D-pantothenonitrile 4′-pyrophosphate) with cysteamine to VIII was enforced to be performed in suspended state in methanol. As a result, the prolonged reaction time seemed to effect the concomitant decomposition of the pyrophosphate bond in VII and VIII. Therefore, a combination of the methods, used by Moffatt and Khorana[10] for the formation of pyrophosphate and Michelson[11]

10) J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, **81**, 1265 (1959); **83**, 663 (1961).
11) A.M. Michelson, *Biochim. Biophys. Acta*, **50**, 605 (1961); **93**, 71 (1964).

for 2′,3′-cyclic phosphate fission was employed to afford guano-CoA in a satisfactory yield of 23.6% from V. Addition of dimethylformamide was effective for solubilizing V in the reaction mixture. The use of ribonuclease T_1 instead of T_2 for fission of 2′,3′-cyclic phosphate was based on the ground that T_1 is the enzyme which hydrolyzes ribonucleic acid specifically to 3′-guanylic acid as a terminus *via* 2′,3′-cyclic phosphate.[12] However, the hydrolyzing rate was considerably low and the larger amounts of T_1 were required than those of T_2 used in the other cases. The structure of guano-CoA thus synthesized in both procedures was confirmed by paper chromatography in the same manner as that employed for inosino-CoA. It was the different point from the cases of CoA and inosino-CoA that chromatography of the crude product on DEAE-cellulose using a linear salt gradient elution gave exclusively the disulfide form of guano-CoA. Guano-CoA thus seems to be more easily oxidized than CoA. Making an additional remark, guanosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (V) used above was synthesized in 90% yield by refluxing guanosine 2′(3′),5′-diphosphate in aqueous tert. BuOH with morpholine and N,N′-dicyclohexylcarbodiimide (DCC). Phosphorylation of guanosine to the diphosphate was effected by standing with cyanoethyl phosphate and DCC in pyridine.

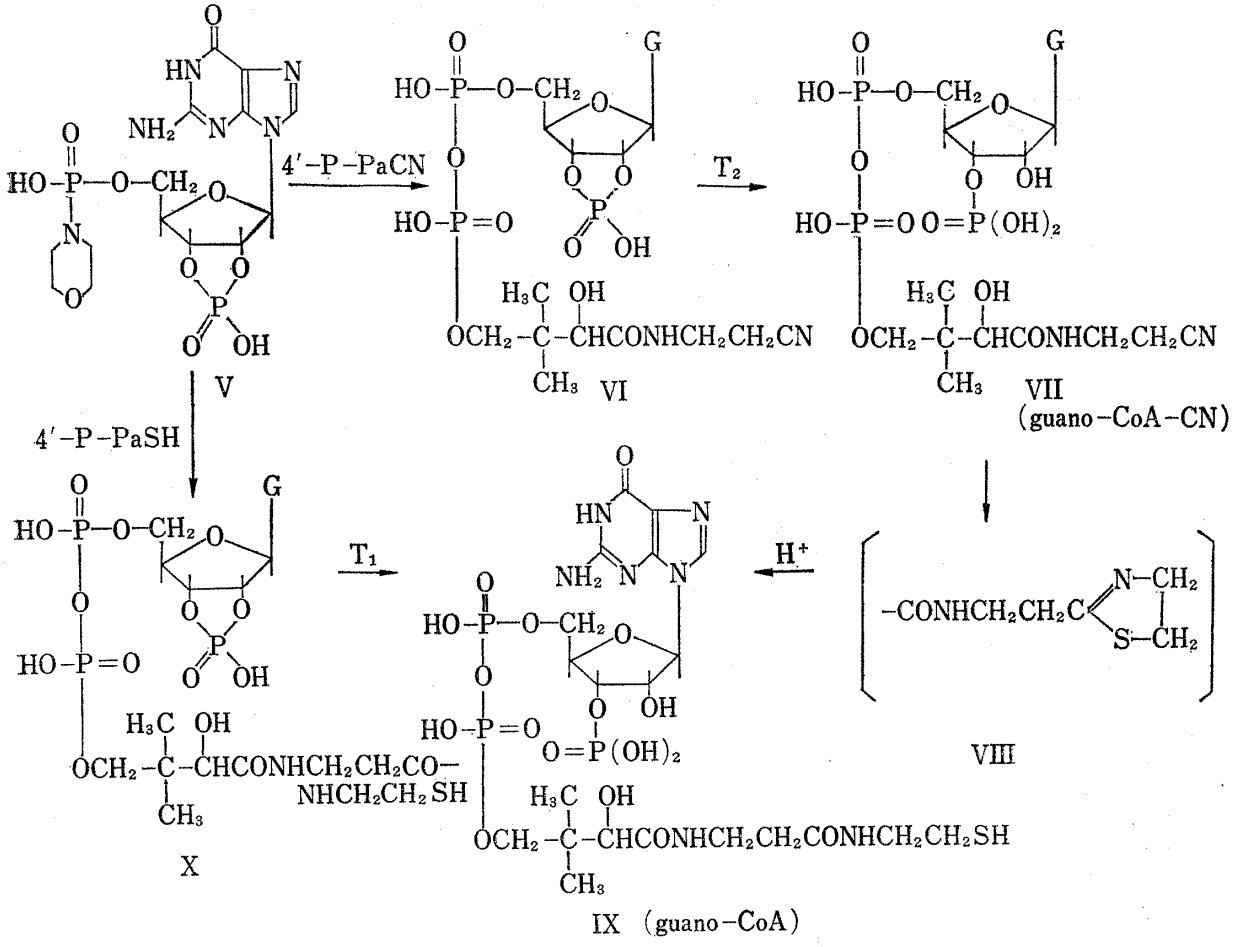

Chart 2

The analogs thus obtained are effectively being used in the biochemical study together with those reported previously[6] and those to be published in a near future. The conclusion on the interaction between phosphotransacetylase and CoA will be reported elsewhere.

12) T. Uchida and F. Egami, "Methods in Enzymology," Vol. XII, ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, N.Y., 1967, p. 228.

Experimental

Paper chromatography (PPC), paper electrophoresis (PEP) and the location of the compounds on chromatograms were carried out by the same method as described previously.[6] The following solvent systems were employed: for PPC, (A) EtOH–0.5M $AcONH_4$ (pH 3.8) (5:2); (B) EtOH–1M $AcONH_4$ (pH 7.5) (5:2); (C) isobutyric acid–1N NH_4OH–0.1N EDTA·2Na (100:60:1.6); (D) saturated $(NH_4)_2SO_4$–0.1M $AcONH_4$ (pH6)–iso-PrOH (79:19:2); (E) PrOH–NH_4OH–H_2O (6:3:1); (F) BuOH–AcOH–H_2O (5:2:3); for PEP, (1) 0.05M $AcONH_4$ buffer (pH 3.5); (2) 0.05M triethylammonium bicarbonate (pH 7.5). *Rf* values and electrophoretic mobilities are summarized in Table I. The quantitative determination of phosphorus was performed by the method of Boltz and Mellon.[13] Inosine and guanosine were determined by UV absorption in pH 7 using $\varepsilon_{248}=12300$[14] and in 0.1N HCl using $\varepsilon_{256}=12400$,[15] respectively.

Table I. Paper Chromatography and Paper Electrophoresis of Different Compounds

Compound	*Rf* in solvent				Electrophoretic mobility in solvent	
	A	B	C	D	1	2
Inosine 5′-phosphate	0.16	0.08	0.31		0.61[a]	0.73[a]
Inosine 2′(3′),5′-phosphate	0.05	0.01	0.22	0.64 (3′,5′) 0.68 (2′,5′)	1.0[a]	1.0[a]
Inosino-CoA–CN	0.22	0.15	0.32			
Inosino-CoA (SH)	0.24	0.09	0.38		0.96[a]	0.81[a]
Guanosine 5′-phosphate	0.19		0.42		0.66[b]	0.80[b]
Guanosine 2′(3′),5′-diphosphate	0.10	0.01	0.19	0.60 (3′,5′) 0.68 (2′,5′)	1.0[b]	1.0[b]
Guanosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate	0.21	0.21				0.78[b]
Guano–CoA–CN	0.16	0.06			0.88[b]	0.91[b]
Guano–CoA (SH)	0.21	0.07	0.36		0.86[b]	0.86[b]
CoA (SH)	0.28	0.12	0.54			

a) mobility relative to inosine 2′(3′),5′-diphosphate
b) mobility relative to guanosine 2′(3′),5′-diphosphate

P^1-Inosine 3′-Phosphate 5′-P^2-D-Pantothenonitrile 4′-Pyrophosphate (II)——To a stirred solution of the Li salt of I[9] (214 mg, 0.262 mmole) in 2N AcOH (10 ml), a solution of $NaNO_2$ (1.6 g) in water (6 ml) was added dropwise. After standing at room temperature for 3 hr, the mixture was neutralized with 2N NaOH and 25% $Ba(OAc)_2$ (1 ml) added along with EtOH (80 ml). The precipitate was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* over P_2O_5 to give the Ba salt of II (264 mg, 92% yield). An aqueous solution of the Ba salt was treated with Dowex 50 (H^+), adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. The residue was dissolved in MeOH (0.5 ml), and acetone was added to give a white powder. Drying over P_2O_5 *in vacuo* gave the Li salt of II (161 mg, 77% yield); UV λ_{max}^{pH2}: 250 mμ, λ_{max}^{pH7}: 249 mμ. IR ν_{max}^{KBr} cm^{-1}: 3390 (OH, NH), 2250 (C≡N), 1680, 1660, 1650 (C=O in hypoxanthine, amide I), 1550 (amide II), 1250 (PO_2^-), 1130, 1090, 1070 (C–O, P–O–C, PO_2^-), 955 (P–O–P). *Anal.* Calcd. for $C_{19}H_{27}O_{16}N_6P_3Li\cdot 5H_2O$: C, 28.55; H, 4.67; N, 10.52; P, 11.72; inosine: P=1:3.0. Found: C, 28.73; H, 4.24; N, 10.40; P, 12.53; inosine: P=1:3.05.

Inosino-CoA (IV)——1) The mixture of the Li salt of II (150 mg, 0.188 mmole) and cysteamine (80 mg, 1.04 mmole) in MeOH (10 ml) was refluxed in N_2 for 7 hr and then concentrated to dryness *in vacuo* to give the crude thiazoline (III) as a white powder which had no band of C≡N group at 2250 cm^{-1} in its IR spectrum.

The crude III was dissolved in water (5 ml) and adjusted to pH 4.7 with HCl. The mixture was heated in N_2 at 60° for 3.5 hr and after cooling adjusted to pH 6. The mixture was concentrated *in vacuo* to a volume of 1 ml of 2-mercaptoethanol (1 ml) was added. The solution was kept at room temperature for 3 hr, diluted with water and passed through a column of Dowex 50 (H^+) (8 ml). The effluent was adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. The residue was dissolved in MeOH (0.5 ml), and acetone (10 ml) was added. The precipitate was dried *in vacuo* to give the crude Li salt of IV (145 mg).

13) D.F. Boltz and M.G. Mellon, *Analyt. Chem.*, **19**, 873 (1947).
14) M.P. Schulman and J.M. Buchanan, *J. Biol. Chem.*, **196**, 513 (1952).
15) R.W. Chambers, J.G. Moffatt, and H.G. Khorana, *J. Am. Chem. Soc.*, **79**, 3747 (1957).

It was dissolved in water (0.5 ml) and adjusted to pH 6 with NH_4OH. 2-Mercaptoethanol (0.5 ml) was then added and the solution kept at room temperature for 3 hr. The mixture was applied to a column (1.2 × 17.5 cm) of DEAE-Sephadex A-25 (Cl^-) and the elution was carried out using a linear salt gradient with 0.003N HCl (1 liter) and 0.3M LiCl in 0.003N HCl (1 liter). Fractions of each 10 ml were collected at a flow rate of 1 ml per min. Inosino-CoA (SH form) was found in the second peak, fractions 86—110 (1390 OD units at 250 mμ, 71% of the total column recovery). The pooled peak was adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. The residue was dissolved in MeOH and precipitated with acetone. Drying over P_2O_5 *in vacuo* gave a white powder of the Li salt of IV (79 mg). The third peak containing inosino-CoA (SS form), fractions 185—200 (234 OD units, 12%) was treated similarly and the resulting Li salt was reduced with 50% aqueous 2-mercaptoethanol to give an additional crop (15 mg). Total yield was 94 mg, 59.5%. IR ν_{max}^{KBr} cm^{-1}: 3400, 1690, 1680, 1655, 1550, 1250, 1130, 1095, 1070, 955. *Anal.* Calcd. for $C_{21}H_{32}O_{17}N_6SLi_3P_3 \cdot 3H_2O$: C, 30.01; H, 4.56; N, 10.00; P, 11.06; inosine:P=1:3.0. Found: C, 29.95; H, 4.46; N, 9.38; P, 11.10; inosine:P=1:2.99.

2) The Li salt of disulfide of CoA (50 mg, 0.063 mmole) was deaminated in 2N AcOH (2.5 ml) with $NaNO_2$ (400 mg). After 3 hr at room temperature, the resulting Ba salt was converted to the Li salt and then reduced with 2-mercaptoethanol to give the Li salt of IV (34 mg, 64% yield).

Inosine 2′(3′),5′-Diphosphate——The deamination of adenosine 2′(3′),5′-diphosphate (300 mg, 0.61 mmole) was carried out in the same manner as with the preparation of II. After neutralization with NaOH and addition of $Ba(OAc)_2$, the Ba salt of inosine 2′(3′),5′-diphosphate (380 mg, 85% yield) was precipitated by the addition of 1 volume of EtOH; UV $\lambda_{max}^{pH\ 2-7}$: 249 mμ; IR ν_{max}^{KBr} cm^{-1}: 1685 (C=O). *Anal.* Calcd. for $C_{10}H_{10}O_{11}N_4Ba_2P_2 \cdot 2H_2O$: C, 16.34; H, 1.92; N, 7.62. Found: C, 16.92; H, 2.41; N, 7.20.

Guanosine 2′(3′),5′-Diphosphate——Guanosine (283 mg, 1 mmole, dried at 110°, 1 mm over P_2O_5) was dissolved in boiling dimethylformamide (DMF, 30 ml) and after cooling, the solution was mixed with a solution of 2-cyanoethyl phosphate[16] (6 mmoles) in anhydrous pyridine (30 ml). Dicyclohexylcarbodiimide (DCC, 3.71 g, 18 mmoles) was added and the mixture left at room temperature for 4 days. Water (15 ml) was added and after 1 hr the mixture was filtered to remove dicyclohexylurea. The filtrate was concentrated to dryness *in vacuo* and a solution of the residue in 25% AcOH (20 ml) was heated at 100° for 30 min. Solvent was removed *in vacuo*, the residue was dissolved in 2N LiOH (15 ml) and the mixture was heated at 80° for 1 hr. After cooling in ice bath, the filtered solution was passed through a column (1.6 × 18 cm) of Dowex 50 (H^+). The eluate and the washing were neutralized with NH_4OH to pH 7.5 and applied to a column (1 × 16 cm) of Dowex 2 X8 (Cl^-). Guanosine monophosphate (1090 OD units at 256 mμ, 8.7%) was eluted with 0.01M LiCl in 0.01N HCl and then guanosine 2′(3′),5′-diphosphate (9920 OD units, 80%) was eluted with 0.1M LiCl in 0.01N HCl. The diphosphate fractions were combined, adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. LiCl was removed by repeated extractions with MeOH–acetone (1:10). Drying over P_2O_5 *in vacuo* gave the Li salt of guanosine 2′(3′),5′-diphosphate as a white powder (240 mg, 48% yield). Its UV absorption spectra were identical with those reported by Michelson.[11] IR ν_{max}^{KBr} cm^{-1}: 3430, 3150 (OH, NH), 1700 (C=O),[17] 1245, 1190, 1100, 1080 (phosphate). *Anal.* Calcd. for $C_{10}H_{13}O_{11}N_5Li_2P_2 \cdot 3H_2O$: C, 24.06; H, 3.84; N, 14.04; P, 12.42; guanosine:P=1:2.0. Found: C, 23.97; H, 3.58; N, 13.66; P, 12.43; guanosine:P=1:2.06.

Guanosine 2′,3′-Cyclic Phosphate 5′-Phosphoromorpholidate (V)——An aqueous solution of the Li salt of guanosine 2′(3′),5′-diphosphate (235 mg, 0.47 mmole) was passed through a column (1 × 5 cm) of Dowex 50 (morpholinium form) and the effluent was evaporated to dryness *in vacuo*. The residue was dissolved in a mixture of water (5 ml), *tert*-BuOH (5 ml) and morpholine (330 mg). To the refluxing solution, a solution of DCC (972 mg, 4.71 mmoles) in *tert*-BuOH (7 ml) was added dropwise during 4 hr and the mixture was refluxed for another 4 hr. After cooling, crystals were removed by filtration and the filtrate was evaporated *in vacuo*. The residue was dissolved in water (20 ml) and the solution was extracted three times with ether. The aqueous solution was evaporated to dryness *in vacuo* and the residue was dissolved in MeOH (1 ml). The addition of ether (40 ml) precipitated a gummy solid which was triturated with dry ether to give a powder of the bis-(4-morpholine N,N′-dicyclohexylcarboxamidinium) salt of V (467 mg, 90.3% yield). *Anal.* Calcd. for $C_{48}H_{82}O_{12}N_{12}P_2 \cdot H_2O$: C, 52.44; H, 7.70; N, 15.30; P, 5.64; guanosine:P=1:2.0. Found: C, 52.69; H, 8.30; N, 14.27; P, 4.85; guanosine:P=1:2.05.

Guano-CoA (IX)——1) A mixture of the pyridinium salt of D-pantetheine 4′-phosphate (0.6 mmole) and the bis-(4-morpholine N,N′-dicyclohexylcarboxamidinium) salt of V (220 mg, 0.2 mmole) was rendered anhydrous by evaporation of added pyridine–DMF (2:1), dissolved in a mixture of anhydrous pyridine (5 ml) and DMF (2.5 ml) and left at room temperature for 24 hr. The solvent was removed by several evaporations *in vacuo* with water and the residue was dissolved in water (10 ml). The pH was adjusted to 7.5 with NH_4OH, and partially purified RNase T_1[12] (16×10^4 units) was added along with 0.2M EDTA (0.2 ml) and 2-mercaptoethanol (2 ml). The mixture was incubated at 37° for 13 hr and concentrated to a volume of 3 ml. 2-Mercaptoethanol (2 ml) was added and the mixture was left overnight. It was diluted

16) G.M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).
17) C.L. Angell, *J. Chem. Soc.*, **1961**, 504.

with water (100 ml) and applied to a column (2.8×33 cm) of DEAE-cellulose (Cl^-). The elution was carried out by a linear gradient using 0.003N HCl (3 liters) and 0.34M LiCl in 0.003N HCl (3 liters). Fractions of 15 ml were collected at a flow rate of 1.5 ml per min and eight peaks were obtained. The eighth peak (665 OD units at 256 mμ, 26.8%) eluted at around 0.25M LiCl concentration was adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. LiCl was removed by repeated triturations with MeOH–acetone (1:10). After being left in 50% aqueous 2-mercaptoethanol (2 ml) overnight, the mixture was evaporated *in vacuo* and the residue was triturated with MeOH–acetone (1:10). Drying over P_2O_5 *in vacuo* gave the Li salt of IX (51.7 mg, 23.6% yield); IR ν_{max}^{KBr} cm^{-1}: 3390 (OH, NH), 1695 (C=O), 1660, 1540 (amide), 1250, 1135, 1100, 960 (phosphate, pyrophosphate). *Anal.* Calcd. for $C_{21}H_{33}O_{17}N_7SLiP_{33}\cdot 17H_2O$: C, 23.11; H, 6.19; N, 8.98; P, 8.51; guanosine:P=1:3.0. Found: C, 23.09; H, 6.08; N, 8.25; P, 8.24; guanosine:P=1:3.13.

2) The Li salt of VII (41.6 mg, 0.05 mmole) was suspended in MeOH (50 ml), cysteamine (21.2 mg, 0.275 mmole) was added and the mixture was refluxed in N_2 for 12 hr during which large amounts of solid remained insoluble. Solvent was removed *in vacuo*, the residue was dissolved in water (5 ml) and the pH was adjusted to 5.0. The mixture was heated at 60° for 5 hr and then worked up as described in the preparation of IV to give the crude Li salt of IX. It was purified by the chromatography on DEAE-cellulose (Cl^-) in the same manner as described above. Following guanosine 3′,5′-diphosphate (140 OD units at 256 mμ, 22.4%) and VII (105 OD units, 16.9%), the disulfide of IX (50 OD units, 8.1%) was eluted. It was treated as described above to give the Li salt of IX (3.8 mg, 7.2% yield).

P^1-Guanosine 3′-Phosphate 5′-P^2-D-Pantothenonitrile 4′-Pyrophosphate (VII)——The reaction of D-pantothenonitrile 4′-phosphate (0.9 mmole) with V (0.3 mmole) was carried out in the same manner as with X. After evaporation of the solvent *in vacuo*, the residue was dissolved in water (6 ml) being adjusted to pH 4.6 with NH_4OH, and 0.16M EDTA (0.3 ml) and RNase T_2[9] (3000 units) were added. The mixture was incubated at 37° for 11 hr, adjusted to pH 6.0 and chromatographed on a column (3.4×30 cm) of DEAE-cellulose (Cl^-) by a linear salt gradient with 0.003N HCl (4.5 liters) and 0.34M LiCl in 0.003N HCl (4.5 liters). Fractions of 22.5 ml were collected every 10 min. The fifth peak containing VII (1230 OD units, 33%) was adjusted to pH 4.5 with LiOH and then worked up in the usual manner to give the trilithium salt of VII (67 mg, 26.9% yield). Its IR spectrum (KBr) was almost identical with that of IX except C≡N band at 2250 cm^{-1}. *Anal.* Calcd. for $C_{19}H_{27}O_{16}N_7Li_3P_3\cdot 6H_2O$: C, 27.45; H, 4.73; N, 11.80; P, 11.18; guanosine:P=1:3.0. Found: C, 27.04; H, 4.29; N, 11.03; P, 10.96; guanosine:P=1:2.93.

The structures of IV and IX were confirmed by alkaline hydrolysis and acid hydrolysis in the same manner as described previously.[6]

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