Synthetic Studies on Phosphorylating Reagent. V. A Convenient Synthesis of 2′,3′-Cyclic Coenzyme A

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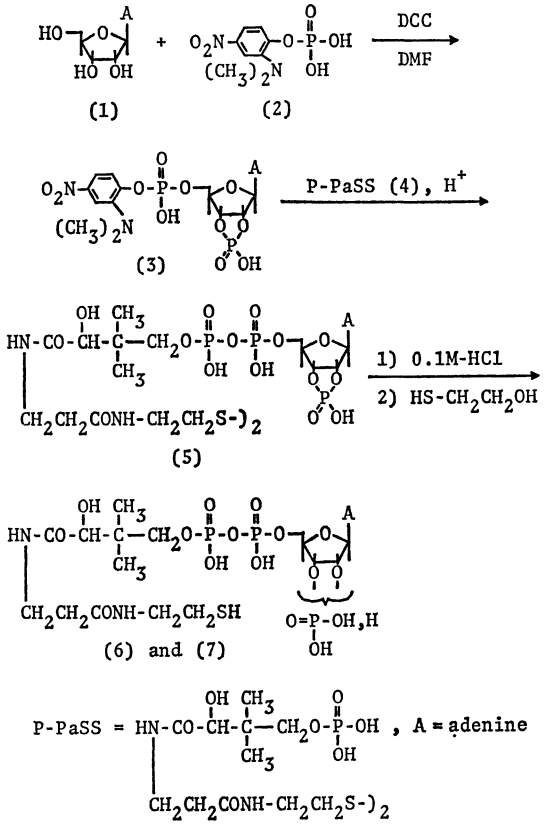
A convenient method has been developed for the preparation of 2′,3′-cyclic Coenzyme A (**5**). It was readily prepared in a good yield from the reaction of adenosine-5′ 2-dimethylamino-4-nitrophenyl phosphate (**3**) with D-pantethine-4′,4″-diphosphate (**4**) in the presence of acetic acid in pyridine. Compound **3** and the diphosphate **4** were prepared in a good yield from the condensation of adenosine (**1**) with 2-dimethylamino-4-nitrophenyl phosphate (**2**) in the presence of dicyclohexylcarbodiimide (DCC) and the phosphorylation of D-pantethine with **2**, respectively.

Phosphate **5** was hydrolyzed with 0.1 M-hydrochloric acid and subsequently reduced with 2-mercaptoethanol to confirm its structure of **5**. One of the products, Coenzyme A (reduced form) (**6**) showed 102% activity with phosphotransacetylase.

It was reported in the previous papers[1–4] that the selective phosphorylation of the hydroxyl group of polyalcohols and amino alcohols including nucleosides, such as adenosine and cytidine *etc.*, was achieved by employing 2-dimethylamino-4-nitrophenyl phosphate (**2**), a new phosphorylating reagent having "an activatable protecting group" to afford the corresponding phosphate esters such as glycerol-1-phosphate, 2-aminoethyl phosphate, adenosine-5′-phosphate and adenosine-3′,5′-cyclic phosphate.

In the present paper, we will describe a convenient preparation of 2′,3′-cyclic Coenzyme A (**5**) using the phosphorylating reagent **2**. There are several papers[5–9] concerning the total synthesis of Coenzyme A (**6**). All methods comprised the synthesis of 2′,3′-cyclic Coenzyme A (**5**) as a precursor of Coenzyme A. However, these methods seem to be impractical because of the difficulties of isolation and purification. Phosphate **5** has not been isolated yet from the reaction mixture and the exact character of **5** has remained unknown. We have attempted the synthesis[10] of **5** using the new phosphorylating reagent **2**. The synthesis of **5** was carried out according to the route shown in Scheme 1.



Scheme 1.

Adenosine-2′,3′-cyclic phosphate-5′ 2-dimethylamino-4-nitrophenyl phosphate (**3**), which acts as a phosphorylating reagent, was prepared by the condensation of the unprotected adenosine (**1**) and the reagent **2** with DCC. Formation of the phosphate **3** was observed in the reaction of **1** and **2** with DCC.[1] Phosphate **3** was obtained as a by-product, the yield being very low. The coupling reaction was reinvestigated in order to find optimum conditions. As a result, phosphate **3** was obtained in quantitative yield when 3 equiv. of the monotriethylammonium salt of **2**, 1 equiv. of adenosine (**1**) and 10 equiv. of DCC were used in the reaction carried out at room temperature in *N,N*-dimethylformamide (DMF). Isolation and purification of product **3** were easily achieved by column chromatography on DEAE-Sephadex according to the usual procedure. Phosphate **3** was obtained in 93% yield as its dilithium salt.

The other component of Coenzyme A (**6**), D-pantetheine-4′-phosphate or D-pantethine-4′,4″-diphosphate (**4**) was necessary for the preparation of **6**. We chose D-pantethine-4′,4″-diphosphate (**4**) as a starting material since the thiol group of D-pantetheine-4′-phosphate would be unfavorable for making the pyrophosphate linkage of **6**. Diphosphate **4** was prepared by Michelson.[6] His method consists of three steps, phosphorylation of D-pantetheine with dibenzyl phosphorochloridate, debenzylation of dibenzyl D-pantetheine-4′-phosphate and oxidation of the thiol group. The procedure seemed to be somewhat intricate. Consequently, a one step synthesis of diphosphate **4** was undertaken with the use of **2**. Thus, the desired diphosphate **4** was readily obtained in 62% yield as its barium salt when a mixture of 1 equiv. of D-pentethine and 2 equiv. of monotriethylammonium salt of **2**

in pyridine was heated under reflux in the presence of 3 equiv. of acetic acid. All the materials including D-pantethine should be thoroughly dried to obtain good results, since this reaction is especially sensitive to the presence of water. The complete drying of D-pantethine was very difficult.

The final step in the preparation of **5** was carried out as follows. The desired pyrophosphate **5** was obtained in 65% yield when 1 equiv. of the triethylammonium salt of **3** was reacted with 3 equiv. of the pyridinium salt of **4** at 50—60 °C for 5 days in pyridine without acid. On the other hand, the yield of **5** increased to 93% when 3 equiv. of glacial acetic acid was added. DMF used in place of pyridine gave the poorest results. The effects of the other acidic media on the yield of **5** are listed in Table 1. The separation of **5** was successfully achieved by eluting a column of DEAE-Sephadex with a linear gradient (0.005—0.30 M) of triethylammonium hydrogencarbonate after applying the reaction mixture to the column and washing with a sufficient amount of water. Analytically pure **5** was obtained as its trilithium salt, a white powder melting at 247—249 °C. The data concerning paper partition chromatography (PPC) and paper electrophoresis (PEP) of compound **5** and the related ones are summarized in Table 2. The structure of **5** was also comfirmed, biologically and chemically, by converting **5** into Coenzyme A (**6**) and *iso*-Coenzyme A (**7**) according to the method of Moffatt and Khorana.[5] Compound **5** (disulfide form) was treated with 0.1 M-hydrochloric acid at room temperature for 1 hr to cleave the cyclic phosphate ring and to reduce subsequently the disulfide linkage with 2-mercaptoethanol at pH 6.0. The products, **6** and **7**, which were isolated as their lithium salts, were chromatographically and electrophoretically identical to a commercially available sample of **6**. The enzymatic assay of the mixture of **6** and **7** by the phosphotransacetylase method[11] showed 102% activity on the basis of **6**.

Table 1. 2′,3′-Cyclic Coenzyme A

Solvent	Acid	Yield (%)
Pyridine	no acid	65
Pyridine	CH_3COOH	93
Pyridine	H_2SO_4	89
Pyridine	CF_3COOH	80
Pyridine	p-$CH_3C_6H_4SO_3H$	78
Pyridine	BF_3-$(C_2H_5)_2O$	65
DMF	BF_3-$(C_2H_5)_2O$	74
DMF	H_2SO_4	48

In conclusion, the key intermediate for the synthesis of Coenzyme A, adenosine-2′,3′-cyclic phosphate-5′ 2-dimethylamino-4-nitrophenyl phosphate (**3**), was easily prepared in one step by the DCC coupling reaction of adenosine (**1**) and the phosphorylating reagent (**2**) in a high yield. A simpler preparation of D-pantethine-4′,4″-diphosphate (**4**) was achieved by using **2**. It was clear that the mild reactivity of phosphate **3** was due to protonation on the nitrogen atom, and **3** reacted with **4** to afford the oxidized form of 2′,3′-cycle Coenzyme A in a good yield.

This method should be useful for the syntheses of other nucleotides having pyrophosphate linkages.

Experimental

Paper chromatography (PPC) was carried out by descending technique using Toyo Roshi No. 51A paper. Solvent systems used were: A, EtOH–0.5 M ammonium acetate buffer (pH 3.8) (5 : 2); B, EtOH–1 M ammonium acetate buffer (pH 7.5) (5 : 2); C, 1-butanol–acetic acid–water (5 : 2 : 3).

Paper electrophoresis (PEP) was performed at pH 7.5 (0.05 M triethylammonium hydrogencarbonate, 900 V/40 cm). The spots of adenosine derivatives on chromatograms were detected with UV lamp (2540 Å). The phsphorus-containing compounds were detected with the Hanes and Isherwood reagent.[12] Chromatographic and electrophoretic mobilities are given in Table 2.

Table 2. Paper chromatography and paper electrophoresis

Compound	R_f in solvent A	B	C	Electrophoretic mobility[a]
Adenosine-2′,3-cyclic phosphate-5′ 2-dimethylamino-4-nitrophenyl phosphate	0.60			0.83
2′,3′-Cyclic Coenzyme A (SS)	0.10	0.08	0.11	1.17
Coenzyme A (SS)	0.07	0.06	0.08	1.20
Coenzyme A (SH)	0.23	0.24	0.23	1.15
Pantethine-4′,4″-diphosphate			0.55	0.50

a) Mobility relative to adenosine-5′-phosphate.

Melting points were determined on a Yamato apparatus, MP-21. The NMR spectra were determined on a Hitachi Perkin-Elmer R-20A instrument (DSS as internal standard). IR spectra were determined on a Shimadzu IR-27G spectrophotometer.

Adenosine-2′,3′-cyclic Phosphate-5′ 2-Dimethylamino-4-nitrophenyl Phosphate (3). A DMF solution (20 ml) of adenosine (**1**) (0.27 g, 1 mmol), DCC (2.06 g, 10 mmol) and pyridinium 2-dimethylamino-4-nitrophenylphosphate (**2**), prepared from ammonium salt of **2** (0.84 g, 3 mmol) by the cation exchange method on Dowex 50W × 8 resin (pyridinium form), was allowed to stand at room temperature for 2 days. After removal of the precipitate by filtration, the filtrate was applied to a column (1.8 × 60 cm) of DEAE-Sephadex (HCO_3^- form) and eluted with a linear gradient of 0.0005 M and 0.1 M of triethylammonium hydrogencarbonate solution (2 l each). The eluates were continuously followed by the UV-monitor (260 nm). The desired compound, adenosine-2′,3′-cyclic phosphate-5′ 2-dimethylamino-4-nitrophenyl phosphate (**3**), was eluted at roughly 0.07 M salt concentration. The combined fractions containing **3** were evaporated to dryness after complete removal of the volatile compounds by co-evaporation of two 10-ml portions of methanol. The residue was dissolved in methanol (5 ml) and a stoichiometric amount of lithium chloride was added. Acetone (20 ml) was added to the methanolic solution to give the crude dilithium salt

of **3** as a yellow precipitate. The recrystallization of the crude product from a mixture of ethanol and acetone gave 0.63 g (93%) of pentahydrate of dilithium salt of **3**. It was confirmed by PPC, PEP and NMR spectroscopy that phosphate **3** was identical to an authentic sample prepared previously.[1]

D-Pantethine-4′,4″-diphosphate (4). To a pyridine solution (30 ml) of D-pantethine (2.77 g, 5 mmol), which was well dried by co-evaporation with three 30-ml portions of pyridine, were added monotriethylammonium 2-dimethylamino-4-nitrophenyl phosphate (**2**) (3.62 g, 10 mmol), acetic acid (1.80 g, 30 mmol) and triethylamine (1.01 g, 10 mmol) and the solution was refluxed for 3 hr. After evaporating the reaction mixture, the residue was dissolved in water (50 ml) and subsequently applied to a column (1.3×75 cm) of Dowex 50W×8 resin (H^+ form). Th column was washed with water (3000 ml) and the aqueous washings were adjusted to pH 7.5 with barium hydroxide. After removal of the precipitate by filtration, the filtrate was evaporated to dryness at a temperature below 40 °C. The residue was treated with ethanol (100 ml) to give 3.05 g (62%) of dibarium D-pantethine-4′,4″-diphosphate (**4**) as white precipitate; NMR (D_2O, ppm): 0.81 (s, 6H, $CH_3\times2$), 0.97 (s, 6H, $CH_3\times2$), 2.50 (t, 4H, $CH_2\times2$), 2.85 (s, 4H, $CH_2\times2$), 3.40—3.63 (m, 12H, $CH_2\times6$), 4.09 (s, 2H, $CH\times2$). IR (Nujol, cm^{-1}): 3280 (OH), 1655, 1563, 1545 (CONH), 1240 (P=O), 1120, 1080 (P–O–C). These data were identical with those of an authentic sample prepared according to Michelson's method.[6]

2′,3′-Cyclic Coenzyme A (5). Typical Procedure: An aqueous solution of dibarium D-pantethine-4′,4″-diphosphate (1.48 g, 3 mmol) was passed through a column of Dowex 50W×8 resin (pyridinium form) and the column was washed with water. The combined eluate and washings were evaporated to dryness. The residue was well dried by co-evaporation with three 10-ml portions of pyridine. After adding the triethylammonium salt of adenosine-2′,3′-cyclic phosphate-5′ 2-dimethylamino-4-nitrophenyl phosphate (**3**) (0.72 g, 1 mmol) to the above residue, the mixture was again dried by co-evaporation with two 25-ml. portions of pyridine. To a solution of the residue in pyridine (25 ml) was added acetic acid (0.18 g, 3 mmol) and the solution was allowed to stand at 50—60 °C for 5 days. After evaporating the solvent, an aqueous solution (500 ml) of the residue was applied to a column (2.4×50 cm) of DEAE Sephadex (HCO_3^-) and the column was washed with water until the optical density of the washings became negligible. The column was then eluted with a linear gradient of 0.005 M and 0.3 M of triethylammonium hydrogencarbonate solution (4 l each) and four hundred 20-ml portions of the fractions were collected. The desired compound, 2′,3′-cyclic Coenzyme A (**5**), was eluted with *ca.* 0.13 M salt solution. The combined fractions, which contained the product **5**, were evaporated to dryness at a temperature below 40 °C. After adding a stoichiometric amount of lithium chloride to a solution of the residue in methanol, the solution was diluted with acetone (40 ml) to give the crude lithium salt of **5**. Repeated reprecipitation of the crude product **5** from methanol and acetone gave 0.77 g (93%) of the pure lithium salt of **5** as white powder; mp 247—249 °C (dec.); NMR (D_2O, ppm): 1.27 (s, 6H, $CH_3\times2$), 1.39 (s, 6H, $CH_3\times2$), 2.75—3.58 (m, 8H, $CH_2\times4$), 3.87—4.29 (m, 18H, $C_5'H_2\times2+C_4'H\times2+CH_2\times6$), 4.37—5.14 (m, 6H, $C_2'H\times2+C_3'H\times2+CH\times2$), 6.11 (d, 2H, $C_1'H\times2$), 8.17 (s, 2H, $C_2H\times2$), 8.52 (s, 2H, $C_8H\times2$). IR (Nujol, cm^{-1}): 3320 (OH), 1655, 1610, 1545 (CONH), 1240 (P=O), 1120, 1080 (P–O–C), 945 (P–O–P). Found: C, 30.07; H, 4.14; N, 11.60%. Calcd for $C_{42}H_{60}N_{14}O_{30}S_2P_6Li_6\cdot8H_2O$: C, 30.08; H, 4.57; N, 11.70%.

Hydrolysis of 2′,3′-Cyclic Coenzyme A (5) with 0.1 M-HCl. An aqueous solution (5 ml) of 2′,3′-cyclic Coenzyme A (disulfide form) (6510 OD units) was allowed to stant at room temperature for 1 hr after its pH had been adjusted to 1.0 with 0.1 M-HCl. The solution was then readjusted to pH 6.0 with ammonium hydroxide. To the neutral solution was added 2-mercaptoethanol (20 ml) and the solution was left overnight. After diluting with water (500 ml), the reaction mixture was applied to a column (3.5×30 cm) of DEAE Sephadex (Cl^- form) and the column was washed with water until the optical densities of the washings became negligible. The column was than eluted with a linear gradient of 2 l of 0.003 M-hydrochloric acid (mixing vessel) and 2 l of 0.003 M-hydrochloric acid containing of 12.72 g (0.3 mol) of lithium chloride (reservoir). Twenty-ml portions of fractions were collected and the elution curve is shown in Fig. 1.

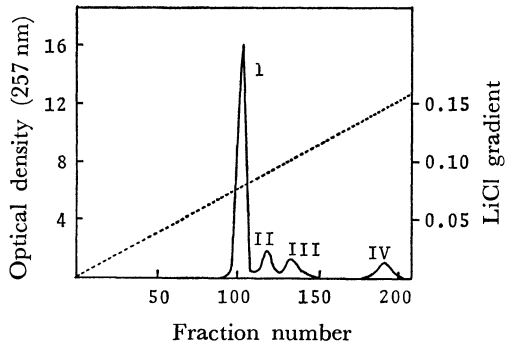


Fig. 1. Chromatography on a DEAE-Sephadex column. Products of the acidic hydrolysis and reduction with 2-mercaptoethanol of 2′,3′-cyclic Coenzyme A (disulfide form).
Peak I, Coenzyme A + *iso*-Coenzyme A; Peak II and III, unidentified; Peak IV, Coenzyme A + *iso*-Coenzyme A (disulfide form).

Peak I (3460 OD units, 49.4%) was a mixture of Coenzyme A and *iso*-Coenzyme A. Peak II (5.8 %) and Peak III (5.5%) could not be identified. Peak IV (320 OD units, 4.6%) was a mixture of oxidized Coenzyme A and *iso*-Coenzyme A. Assay of the mixture of Coenzyme A and *iso*-Coenzyme A, which was obtained from Peak I, by the phosphotransacetylase method[11] using purified commercially available Coenzyme A as the standard proved to be 102% activity on the basis of Coenzyme A, as the ratio of Coenzyme A and *iso*-Coenzyme A was presumed to be 1 : 1.[5]

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10) The usual methods employ the hydrolysis of **5** with dilute hydrochloric acid to obtain Coenzyme A (**6**). However, this usually gives a mixture of Coenzyme A (**6**) and *iso*-Coenzyme A (**7**), which has no activity to the enzyme, the ratio of the two products being about 1 : 1.
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