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Studies on the Vilsmeier-Haack Reaction. III.[1] Synthesis of Cytidine Diphosphate Choline

KIYOMI KIKUGAWA, MOTONOBU ICHINO and TAKESHI KAWASHIMA

Research Laboratory, Division of Fermentation and Chemical Products, Kohjin Co., Ltd.[2]

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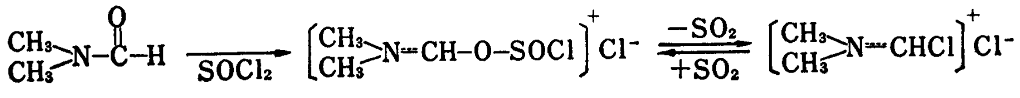
Cytidine diphosphate choline (CDP-choline), one of the nucleotide coenzymes having pyrophosphate linkages, was synthesized in a fairly good yield by the direct condensation of cytidine-5′ monophosphate (5′-CMP) and choline phosphate (P-choline) by means of thionyl chloride combined with dimethylformamide (Vilsmeier–Haack reagent).

Nucleotide coenzymes have been synthesized (a) by direct condensation of phosphoric acids with dicyclohexylcarbodiimide (DCC),[3] or (b) *via* intermediates of nucleoside phosphorochloridates,[4] phosphoromorpholidates,[5] α-pyridyl phosphates,[6] diphenylphosphoric anhydrides[7] and S-ethyl phosphorothioates.[8] In the previous paper,[9] we reported that the *p*-toluenesulfonyl (or methanesulfonyl) chloride-dimethylformamide complex is conveniently used in the direct synthesis of a nucleotide coenzyme such as cytidine diphosphate choline (CDP-choline).[10] The chemical syntheses of this coenzyme had been investigated by Kennedy[11] who obtained it by direct condensation of cytidine-5′ monophosphate (5′-CMP) with choline phosphate (P-choline) in the presence of DCC, and by others[12] *via* several intermediates.

This time, we attempted a direct condensation of two phosphate groups, 5′-CMP and P-choline, by means of Vilsmeier–Haack reagents,[13] and this paper will show that one of these reagents is conveniently used as well as the *p*-toluenesulfonyl (or methanesulfonyl) chloride-dimethylformamide complex for the synthesis of CDP-choline.

It has been reported that thionyl chloride can combine with N,N′-dimethylformamide (DMF) to form the intermediate complex (I) and removal of sulfur dioxide from I under



I II

Chart 1

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1) Part II: K. Kikugawa and T. Kawashima, *Chem. Pharm. Bull.* (Tokyo), **19**, 2629 (1971).
2) Location: *Higashihama, Saiki, Oita.*
3) H.G. Khorana and A.R. Todd, *J. Chem. Soc.*, **1954**, 3733.
4) S.M.H. Christie, G.W. Kenner and A.R. Todd, *J. Chem. Soc.*, **1954**, 46.
5) J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, **84**, 649 (1961).
6) W. Kampe, *Chem. Ber.*, **98**, 1031 (1965).
7) A.M. Michelson, *Biochim. Biophys. Acta*, **91**, 1 (1964).
8) *a*) A.F. Cook, M.J. Holman and A.L. Nussbaum, *J. Am. Chem. Soc.*, **91**, 1522 (1969); *b*) T. Hata and I. Nakagawa, *J. Am. Chem. Soc.*, **92**, 5516 (1970).
9) K. Kikugawa and M. Ichino, *Chem. Pharm. Bull.* (Tokyo), **19**, 1011 (1971).
10) E.P. Kennedy and S.B. Weiss, *J. Biol. Chem.*, **222**, 193 (1956).
11) E.P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956).
12) *a*) Y. Sanno and K. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **10**, 231 (1962); *b*) R. Letters and A.M. Michelson, *Bull. soc. chim. biol.*, **45**, 89 (1963); *c*) T. Tanaka, H. Tanaka, T. Saito and T. Ishida, *Yakugaku Zasshi*, **85**, 863 (1963).
13) *a*) A. Vilsmeier and A. Haack, *Chem. Ber.*, **60**, 119 (1927); *b*) H.H. Bosshard, R. Mory, M. Schmid and H. Zollinger, *Helv. Chim. Acta*, **42**, 1653 (1959).

reduced pressure or by heating affords the crystalline complex (II), which can be reversibly converted into I by treatment with anhydrous sulfur dioxide.[1,13b] There have been few attempts to prepare pyrophosphate bonds with these complexes,[14] although several studies to prepare phosphodiester linkages have been done with the complex (II).[15] We attempted to prepare pyrophosphate linkages using complex (I) and complex (II). It was found that in the condensation of 5′-CMP (III) and P-choline (IV) using the complex (I), CDP-choline (VI) was obtained in a good yield of 50—60%, whereas the expected product (VI) could not be obtained in the condensation using the complex (II). Thus, 5′-CMP (III) was treated with P-choline (IV) which was dissolved in a solution of DMF containing the complex (I) prepared by the direct mixing of thionyl chloride and DMF. After one hour's reaction at room temperature the reaction mixture was added with water, stirred for an hour, and it was subsequently applied to a column of Dowex 1 (formate). The column was eluted with formic acid to obtain the ultraviolet (UV) absorbing peak which afforded the hygroscopic powder of CDP-choline (VI) in a yield of about 60%. The same result was obtained when the complex (I) which was prepared by the reaction of the complex (II) with an equimolar amount of sulfur dioxide was used.

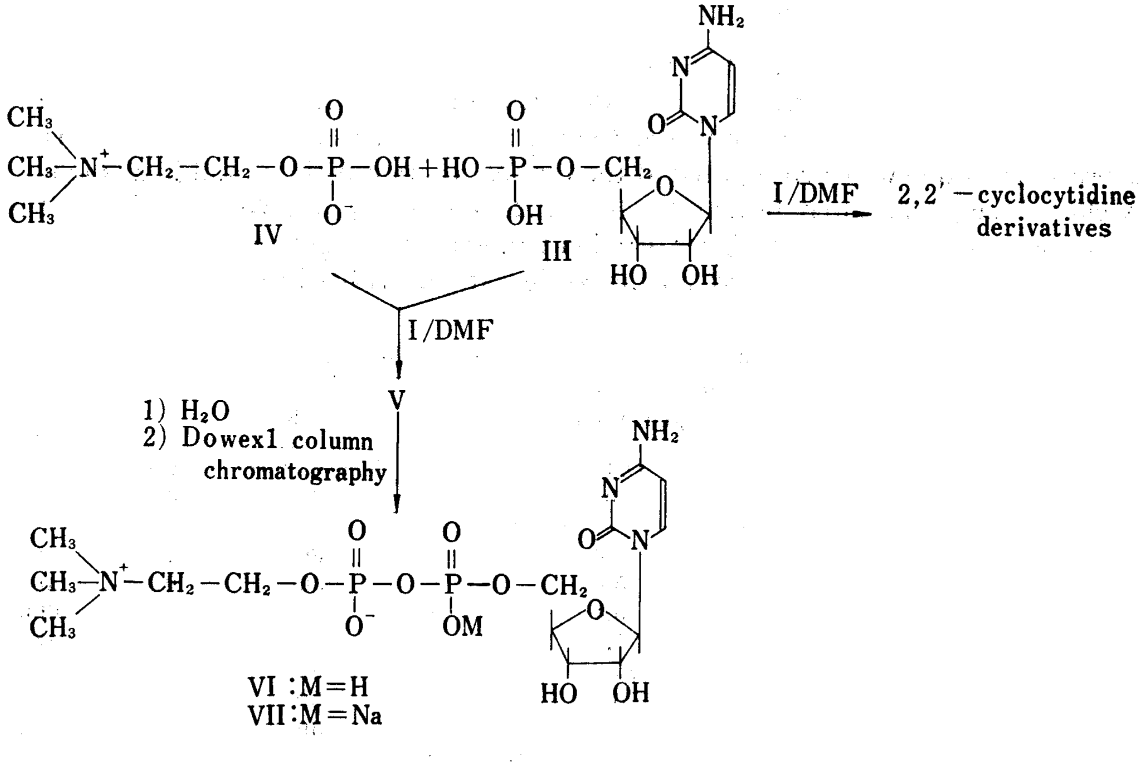

Chart 2

Paper chromatography and paper electrophoresis of the product (VI) showed a single spot in every solvent or buffer system, and *Rf* values and mobilities of the product were quite identical with those of the authentic sample of CDP-choline prepared accoring to the reported methods.[9,12] Other physicochemical properties of the compound (VI) were identical with

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[14] M. Ikehara and H. Uno (ref. 15b) detected TppT in the condensation of 3′-O-acetylthymidine-5′ monophosphate and 5′-*O*-trityl-thymidine by means of the complex (II).

[15] *a*) T.M. Jacob and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964); *b*) M. Ikehara and H. Uno, *Chem. Pharm. Bull.* (Tokyo), **13**, 974 (1965); *c*) F. Cramer, S. Rittner, W. Reinhard and P. Desai, *Chem. Ber.*, **99**, 2252 (1966).

those of the authentic sample.[9] Elemental analysis of the crystalline monosodium salt (VII) prepared from VI was in good accordance with the calculated value, and the signal at δ 3.25 ppm (9 protons) of the nuclear magnetic resonance (NMR) spectrum of VII indicated the presence of the choline moiety (Fig. 1).

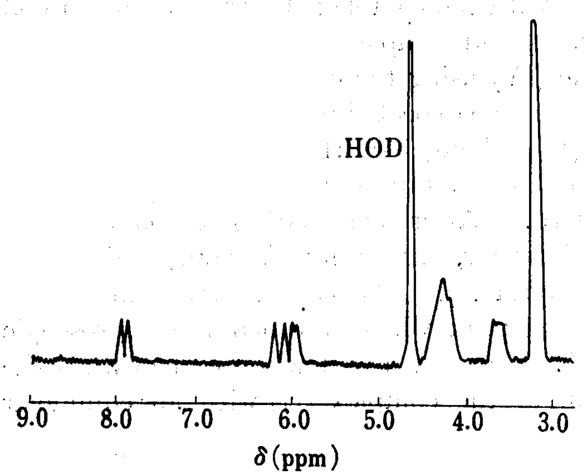

Fig. 1. NMR Spectrum of CDP-choline Monosodium Salt (VII) in D_2O

Several reaction conditions were investigated with the complex (I) prepared by direct mixing of thionyl chloride and DMF to obtain the highest yield of the compound (VI) (see Table I). A small excess of thionyl chloride to P-choline (IV) was found best to obtain the highest yield of the product (VI), and a large excess of the reagent reduced the yield of the product (Exp. No. 1,2). A large excess of the reagent, however, was found effective when P-choline (IV) contained a large amount of water (Exp. No. 2). The yield of the product was influenced by the amount of DMF, and it was shown that the minimum amount of DMF which could maintain the reaction mixture homogeneous gave the best result (Exp. No. 3). Although the presence of pyridine in the reaction mixture did not influence the yield of the product, calcium ion prevented the reaction (Exp. No. 4). The conditions of the condensation reaction were investigated at the various reaction periods, and it was found that the yields of the product were best at 20—30° and for one hour (Exp. No. 1,5 and 6). The low yields of the product (VI) at the higher reaction temperatures might probably due to the conversion of I to II[1] which is inactive for the condensation. It has been demonstrated by us[16]

TABLE I. Reaction Conditions in the Condensation of 5′-CMP (III) and P-choline (IV)[a]

Exp. No.	Amount of the reagent used				Reaction		Yield (%) of VI
	H_2O[b] mmole	$SOCl_2$ mmole (ml)	DMF[c] ml	Base mmole	Temperature °C	Time hr	
1	1.0	11.0(0.79)	5.4	—	30	1.0	59.5
2		30.0(2.14)	5.4	—	30	1.0	0
	12.0	30.0(2.14)	5.4	—	30	1.0	43.0
	12.0	11.0(0.79)	5.4	—	30	1.0	0
3	1.0	11.0(0.79)	75	—	30	1.0	36.2
	1.0	11.0(0.79)	150	—	30	1.0	22.5
4	1.0	11.0(0.79)	5.4	15.0 (pyridine)	30	1.0	56.0
	4.0[d]	11.0(0.79)	5.4	10.0[d] (calcium)	30	1.0	0
5	1.0	11.0(0.79)	5.4	—	30	0.5	54.0
	1.0	11.0(0.79)	5.4	—	30	3.0	49.5
6	1.0	11.0(0.79)	5.4	—	20	1.0	53.5
	1.0	11.0(0.79)	5.4	—	40	1.0	46.0
	1.0	11.0(0.79)	5.4	—	50	1.0	5.0

a) 5′-CMP(3.1 mmoles) was treated with 10 mmoles of P-choline.
b) Water content in the P-choline preparation was listed in the column.
c) Additive amounts of DMF were given contained in the P-choline preparation and thionyl chloride-DMF mixture.
d) P-choline calcium salt tetrahydrate was used.

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[16] a) K. Kikugawa and M. Ichino, *Tetrahedron Letters*, **1970**, 867; b) K. Kikugawa and M. Ichino, *J. Org. Chem.*, in press.

that cytidine could react with the complex (I) undergoing intramolecular cyclization of 2 and 2'-positions. Although 5'-CMP (III) suffered the similar reaction with I,[17] it did not undergo such an intramolecular cyclization in the presence of P-choline (IV). CDP-choline preparation thus obtained was examined whether it was contaminated with any compounds derived from the 2,2'-cyclized products, and it was found free from such compounds.

Table II. Conversion of the Unstable Compound (V) to VI

Treatment of the reaction mixture with water min	Bisulfite ion remained[a)] %	Yield (%)[b)] V	 VI
5	30	34.0	22.5
20	0	12.0	44
60	0	0	57.5

a) estimated by titration with 0.1N iodine

b) The yields of V and VI were estimated by the $\mathrm{TOD}_{260\,m\mu}$ (pH 1) of the fractions against that of the initial mixture.

Aqueous treatment of the reaction mixture was necessary to remove bisulfite ion derived from the reagent and it was also effective to obtain the highest yield of the product (VI). It was found that by the one hour treatment of the condensation mixture with water VI was isolated in a yield of 50—60%, but VI was isolated in a lower yield of 20—30% by an insufficient treatment of the same reaction mixture. In an insufficient treatment of the reaction mixture with water, about 30—40% of the UV absorbing substance which could not absorb to the ion exchange column was obtained. Table II shows that the unstable product (V) which was initially formed by the condensation, though not isolated, was gradually hydrolyzed into the stable anionic product (VI).

Experimental

Methods——Paper chromatography was performed on Toyo Roshi No. 51 A paper using solvent systems, (1) iso-PrOH–conc.NH_4OH–H_2O (7:1:2, V/V), (2) EtOH–0.5M NH_4OAc (5:2, V/V), (3) iso-AmOH–HCOOH–H_2O (3:2:1, V/V), (4) *n*-BuOH–H_2O (84:16, V/V) and (5) 5M NH_4OAc–0.5M EDTA·Na_4–satd. $Na_2B_4O_7$–EtOH (12:0.3:48:132, V/V). Paper electrophoresis was performed on Toyo Roshi No. 51 A paper using buffer systems, (A) 0.02M phosphate buffer (pH 7.5), (B) 0.05M acetate buffer (pH 4.5) and (C) 0.05M borate buffer (pH 6.0) at 1000 V/25 cm.

Water content was determined by Karl Fisher method, and NMR spectra were taken with Varian T-60 spectrometer.

Other methods were same as previously mentioned.[9]

Intestinal alkaline phosphomonoesterase was perchased from Tokyo Kasei Kogyo Co., Ltd.

P-choline (IV)——P-choline (IV) was prepared from its calcium salt according to the previously mentioned method.[9] The gummy residue (3.1 g) which contained 10 mmoles of P-choline (IV), 1 mmole of water and 0.9 g of DMF was obtained, and it was directly used in the next synthetic procedures.

CDP-choline (VI)——Method A, with Complex (I) Prepared by the Direct Mixing of Thionyl Chloride and DMF: The gummy mixture containing 10 mmoles of P-choline (IV) was dissolved in a mixture of 0.79 ml (11.0 mmoles) of thionyl chloride and 4.5 ml of DMF at room temperature during 10 min. 5'-CMP (III) (1.0 g, 3.1 mmoles) was immediately added to the viscous solution and the mixture was stirred at room temperature for one hour. The reaction mixture was then added with 500 ml of water, and stirred

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[17] 5'-CMP (1.0 g) was reacted with the mixture of 0.79 ml of thionyl chloride and 4.5 ml of DMF at room temperature. Paper chromatography (iso-PrOH–1M NH_4OAc (pH 4.0), 7:3) revealed two main spots having *Rf*'s of 0.66 and 0.21, neither of which corresponded to the starting 5'-CMP. UV-spectra of the aqueous extracts of the spots showed λ_{max} (pH 1—6), 233 and 264 m*μ*, the characteristic absorption maxima of 2,2'-cyclocytidine derivatives.

vigorously for one hour to remove sulfur dioxide and hydrolyze the initially formed product (V) completely. After pH of the aqueous mixture was adjusted to 9.5 with conc.NH_4OH, it was submitted to a column (3 × 50 cm) of Dowex 1 × 4 (formate, 200—400 mesh). The column was washed with water to remove P-choline (IV) and was eluted with 0.01M formic acid to obtain the UV-absorbing peak containing the final product (VI). Other peaks culd not be obtained by the elution with formic acid, and higher polymeric substances were eluted when the column was washed with 2N NaOH.

The peak obtained in a yield of 60% estimated by the total optical density (TOD) at 280 mμ and pH 1 against that of the initial mixture was coevaporated with ethanol to dryness and afforded 0.95 g (yield, 57% against 5′-CMP (III)) of white crystalline powder of the product (VI). The product (VI) was identified with CDP-choline as follows.[9)]

Paper chromatography, *Rf*: 0.22 (solvent, 1), 0.17 (solvent, 2), 0.43 (solvent, 3). Paper electrophoresis, the mobility relative to 5′-CMP; +0.41 (buffer system, A), +0.29 (buffer system, B). The spots had the same properties as those of the authentic sample.[9),12c)] UV: λ_{max} (pH 1) 280, λ_{min} (pH 1) 242, λ_{max} (pH 7) 272, λ_{min} (pH 7) 250 mμ. ε 280 (pH 1)/ε 260 (pH 1) = 2.12, ε 250 (pH 1)/ε 260 (pH 1) = 0.44. Phosphorus (2.03 gram atom) was found in 1.00 mole of VI determined by ε 280 (pH 1); 12800. $[\alpha]_D^{25^\circ} = +19.0^\circ$ (C; 0.86 in H_2O, based on the dried matter).

The product (VI) contained 10±5% of water determined by loss on drying.

The product (VI) (10 mg) was dissolved in 0.5 ml of 1N HCl and heated at 100° for one hour. Paper chromatography (solvent, 1,3) of the hydrolysate revealed that the product was degradated into 5′-CMP (III) and P-choline (IV), and the UV absorbing substance corresponding to 5′-CMP was quantitatively obtained by use of the column of Dowex 1 (formate).[18)] The substance was then incubated with alkaline phosphomonoesterase in 0.01M Tris-HCl buffer (pH 9.0) at 37° overnight. The incubated mixture was submitted to paper chromatography (solvent, 4 and 5),[19)] paper electrophoresis (buffer system, C)[20)] and to a column of Dowex 1 (OH^-)[21)] which have been known useful to distinguish arabinosyl cytosine derivatives from cytidine. Only the spots and the peak corresponding to cytidine were obtained and those corresponding to arabinosyl cytosine derivatives were not detected in every solvent system, buffer system and in the column chromatography. Thus, the product (VI) was found free from arabionsyl cytosine derivatives.

Method B, with Complex (I) Prepared by the Complex (II) and Sulfur Dioxide: A mixture of 0.79 ml (11.0 mmoles) of thionyl chloride and 0.80 ml (11.0 mmoles) of DMF was evaporated *in vacuo* at 40—50° to afford 1.41 g (11.0 mmoles) of the crystalline complex (II). It was then dissolved in 4.5 ml of DMF containing 0.70 g (11.0 mmoles) of anhydrous sulfur dioxide, and to the mixture was added 10 mmoles of P-choline (IV). It was shaken at room temperature for 10 min. to afford the viscous solution. 5′-CMP (III) (1.0 g, 3.1 mmoles) was immediately added to the viscous solution and the reaction was performed similarly as in method A. Crystalline powder of CDP-choline (VI) was isolated in a yield of 54%.

CDP-choline Monosodium Salt (VIII)——The product (VI) (500 mg) was converted into crystalline monosodium salt (VII) as was described in the previous paper,[9)] and 450 mg of VII was obtained. Analysis; calculated for $C_{14}H_{25}O_{11}N_4NaP_2 \cdot 4H_2O$, C, 28.87; H, 5.71; N, 9.62; Na, 3.95; H_2O, 12.38%, and found C, 28.63; H, 5.65; N, 9.06; Na, 4.4; H_2O, 11.99%. $[\alpha]_D^{20^\circ} = +12.5^\circ$ (c = 1.0 in H_2O, based on the dried matter). NMR spectrum of VII taken in D_2O with an internal standard of DSS showed the sharp signal at δ 3.25 ppm (9 protons) indicating the presence of the methyl groups of choline moiety (Fig. 1).

Estimation of the Yield of the Product (VI)——Several reaction conditions were examined to obtain the highest yield of the product (VI). 5′-CMP (III) (1.0 g, 3.1 mmoles) was reacted with 10 mmoles of P-choline (IV) which was dissolved in the mixture of thionyl chloride and DMF at room temperature during 10 min.

The determination of the yield of the compound (VI) was as follows. After an aliquot (one tenth) of each reaction mixture was added with 50 ml of water ($TOD_{280m\mu}$(pH 1), 4000), stirred for one hour and made alkaline (pH 9.5) with NH_4OH, it was submitted to a column (1.7 × 50 cm) of Dowex 1 × 4 (formate) (200—400 mesh). The column which was washed well with water was eluted with 0.01M formic acid to obtain the peak (600—900 ml fraction). The yield of the compound (VI) was estimated by the $TOD_{280m\mu}$(pH 1) of the peak against that of the initial $TOD_{290m\mu}$(pH 1). The results were listed on Table I.

Aqueous Treatment of the Reaction Mixture——5′-CMP (III) (1.0 g, 3.1 mmoles) was reacted with 10 mmoles of P-choline in the presence of thionyl chloride and DMF. After one hour's reaction at room temperature, the reaction mixture was added with 500 ml of water. Each one tenth of the aqueous mixture was stirred vigorously for the indicated period, and adjusted to pH 9.5 with NH_4OH. It was then applied to Dowex 1 (formate) column mentioned above and eluted. The flow rate of the elution was kept constant at 5 ml/min. The substance (V) which could not absorb to the column presented in an insufficiently treated

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18) W.E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).
19) A.W. Schrecker, J.A.R. Mead and M.J. Urshel, *Biochem. Pharmacol.*, **15**, 1443 (1966).
20) J.F. Codington, R. Fecher and J.J. Fox, *J. Am. Chem. Soc.*, **82**, 2794 (1960).
21) C.A. Dekker, *J. Am. Chem. Soc.*, **87**, 4027 (1965).

aqueous mixture. Table II indicates that the bisulfite ion was removed very rapidly but the complete conversion of the unstable product (V) into the final product (VI) was not observed until the aqueous mixture was treated for one hour.

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