# $P^1$-(GUANOSINE-$5^2$)-$P^2$(2-AMINOETHYL-1) PYROPHOSPHATE AS MODEL SUBSTANCE OF A COVALENTLY COUPLED AMINOALKYL GROUP AT THE 5'-TERMINAL NUCLEOTIDE OF TRANSFER RNA*

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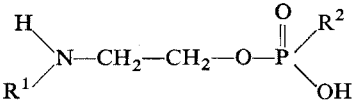
Received September 4th, 1974

2-Aminoethyl-1-phosphate (*Ia*) and 6-aminohexyl-1-phosphate (*IIIa*) were converted to the N-dimethoxytrityl derivatives *Ib* and *IIIb* and then (by the action of carbonyldiimidazole) to 2-(dimethoxytritylamino)ethyl- (*Ic*) and 6-(dimethoxytritylamino)hexyl-1-phosphoimidazolidate (*IIIc*), resp. Reaction of compound *Ic* with cetyltrimethylammonium salt of guanosine 5'-phosphate affords (after removal of the dimethoxytrityl group) $P^1$-(guanosine-5')-$P^2$-(2-aminoethyl-1) pyrophosphate (*IIb*). The imidazolidate *IIIc* affords with the cetyltrimethylammonium salt of transfer RNA (yeast) a product which is coupled after removal of the dimethoxytrityl group, to Sepharose-4B activated with bromocyan.

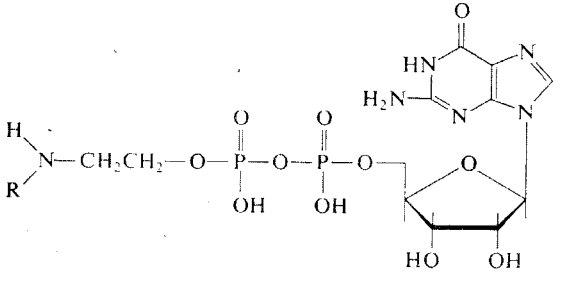
Biopolymers are immobilised by the covalent coupling to Sepharose previously modified by the action of bromocyan[1]. The process is widely used in the case of proteins which always contain amino groups necessary in the coupling reaction. The nucleic acids cannot be used for the coupling reaction in the original form since they lack a free amino group. With transfer RNA, the coupling may be performed through the free amino group of aminoacyl-t-RNA. Thus, isoleucyl-t-RNA was coupled to Sepharose 4-(bromoacetylamino)butyl derivative[2]. After the periodate oxidation of the *cis*-diol system, the 3'-end of t-RNA may be coupled to a hydrazide group-bearing Sepharose[3]. t-RNA could be immobilised without affecting its acceptor end by a process which would involve covalent coupling of t-RNA to the carrier through the 5'-end[4,5]. In most sequenced t-RNA, this end is formed by guanosine 5'-phosphate while cytidine 5'-phosphate plays this role less frequently. From the chemical standpoint, the mildest method how to modify the 5'-end of t-RNA consists in the formation of a pyrophosphate group. As shown by Raj-Bhandary and coworkers[6], the reaction of $^{14}$C-methylphosphomorpholidate with cetyltrimethylammonium salt of t-RNA leading to a mixed diester of pyrophosphoric acid, does not affect the acceptor activity. An analogous procedure has been used by Yang and Söll[7] for the introduction of fluorescent groups into the 5'-terminal t-RNA.

* Part LIII in the series Oligonucleotidic Compounds; Part LII: This Journal *40*, 1043 (1975).

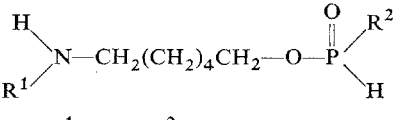
For the t-RNA coupling a derivative was required which would contain a free amino group. In the pyrophosphate approach, an aminoalkylphosphomorpholidate could be used as the starting material. The attempted preparation of a morpholidate from 2-aminoethyl-1-phosphate failed probably because of the interference of the free amino group. The choice of potential protecting groups of the amino function was considerably limited by the sensibility of the t-RNA molecule. On the basis of an information[8] on a sufficient stability of t-RNA in 7M urea even at pH 2, an approach was looked after which would make possible the final deblocking of the amino group under similar conditions. The dimethoxytrityl residue represents a sufficiently acidolabile group and its removal from the amino group is easier than that from



*Ia*; $R^1 = H$, $R^2 = OH$
*Ib*; $R^1$ = dimethoxytrityl, $R^2 = OH$
*Ic*; $R^1$ = dimethoxytrityl, $R^2$ = 



*IIa*; R = dimethoxytrityl
*IIb*; R = H



*IIIa*; $R^1 = H$, $R^2 = OH$
*IIIb*; $R^1$ = dimethoxytrityl, $R^2 = OH$
*IIIc*; $R^1$ = dimethoxytrityl, $R^2$ = 

a hydroxylic function. As shown by dimethoxytritylation of 2-aminoethyl-1-phosphate in pyridine, the reaction is not complete. On the other hand, the reaction is successful when performed in dimethylformamide starting from the triethylammonium salt of compound *Ia*. The thus-obtained 2-dimethoxytritylaminoethyl-1-phosphate (*Ib*) is stable in the form of the triethylammonium salt. The attempted conversion of this substance to the morpholidate by a general method[9] was however accompanied by removal of the dimethoxytrityl group even when a considerable excess of morpholine was used.

An alternative method in the preparation of mixed diesters of pyrophosphoric acid consisted in the synthesis by means of phosphoimidazolidates which are accessible by reaction of the phosphomonoester with carbonyldiimidazole[10,11]. The reaction of the triethylammonium salt of compound *Ib* with carbonyldiimidazole in dimethylformamide was checked by thin layer chromatography; in this manner the imidazolate was shown to be formed in a quantitative yield within two days. After decomposition of excess carbonyldiimidazole by the addition of methanol and evaporation of dimethylformamide, the thus-prepared 2-dimethoxytritylaminoethyl-1-phosphoimidazolidate (*Ic*) was treated with the cetyltrimethylammonium salt of guanosine 5′-phosphate in dimethyl sulfoxide. The resulting $P^1$-(guanosine-5′)-$P^2$-(2-dimethoxytritylaminoethyl-1) pyrophosphate (*IIa*) was separated from the excess compound *Ic* by preparative thin-layer chromatography. Detritylation with 80% aqueous acetic acid and preparative paper chromatography yielded $P^1$-(guanosine-5′)-$P^2$-(2-aminoethyl-1) pyrophosphate (*IIb*); the snake venom phosphodiesterase degradation of compound *IIb* affords guanosine 5′-phosphate. This model experiment has shown that the preparation of a mixed ester of an amino alcohol and t-RNA could be realised. The long-termed treatment of t-RNA with a phosphoimidazolidate could be accompanied by a side-reaction consisting in the formation of phosphoamidates on amino groups of basic components[11]. In order to verify the interference of this side reaction if any, cytidine, adenosine, and guanosine were treated with the imidazolidate *Ic* in dimethyl sulfoxide, the reaction mixtures processed with 80% aqueous acetic acid, and subjected to paper chromatography: only the starting nucleosides were present, *i.e.*, the transamidation did not take place in the present case.

In coupling applications of t-RNA aminoalkyl pyrophosphates to gels. a greater distance between the amino group and the biopolymer molecule would be advantageous from steric reasons. 6-(Dimethoxytritylamino)hexyl-1-phosphoimidazolidate (*IIIc*) was therefore prepared from 6-amino-1-hexanol *via* the corresponding phosphate *IIIa* and used in modifications of 5′-terminal nucleotides in transfer RNA (yeast). Thus, the cetyltrimethylammonium salt of t-RNA was kept in a dimethyl sulfoxide solution of the imidazolidate *IIIc* for a week, the excess reagent removed, and the residual product subjected to the action of formic acid in 7M urea (pH 2·3) to remove the dimethoxytrityl group. The bromocyan-modified Sepharose 4B column bound

36% of the corresponding product which did not exhibit any acceptor activity[5] in contrast to the earlier reported modified t-RNA[6,7].

## EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol $UV_{254}$ silica gel foils (Kavalier Glassworks, Votice, Czechoslovakia). Preparative thick-layer chromatography was carried out on loose layers of a fluorescent indicator-containing silica gel (produced by Service Laboratories of the Institute). Paper chromatography was effected on paper Whatman No 1 (preparative runs on Whatman No 3 MM) by the descending technique. Solvent system $S_1$, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2). Electrophoresis was performed on paper Whatman No 1 dipped in tetrachloromethane using 0·05M triethylammonium hydrogen carbonate (pH 7·5). Unless stated otherwise, the $R_F$ values refer to Silufol.

### 2-Aminoethyl-1-phosphate (*Ia*)

The earlier reported[11] method was modified as follows. A mixture of phosphoric acid (65 g) and phosphorus pentoxide (50 g) is stirred until a homogeneous solution is obtained. Ethanolamine (8 ml) is added to the cooled solution and the whole is heated at 65°C for 2 h. Water (500 ml) is added, the mixture heated at 100°C for 30 min, neutralised while hot with hot saturated aqueous barium hydroxide (phenolphthalein paper as indicator, pink colour). The mixture is filtered while hot through a Celite layer and the material on the filter is washed with hot water (2 l). The filtrate and washings are combined, concentrated under diminished pressure to the volume of about 300 ml, and the concentrate diluted with ethanol (600 ml) to deposit a sirup which is dissolved in water. The aqueous solution is precipitated with the equivalent amount of dilute sulfuric acid, the barium sulfate is removed by centrifugation, and the supernatant is evaporated under diminished pressure to afford 9·5 g of a crystalline product *Ia*, m.p. 235°C.

### 2-(Dimethoxytritylamino)ethyl-1-phosphate (*Ib*) (Triethylammonium Salt)

A solution of 2-aminoethyl-1-phosphate (*Ia*; 0·7 g) in 50% aqueous ethanol (20 ml) and triethylamine (2 ml) is evaporated to dryness and the residue is coevaporated with three 5 ml portions of 1 : 1 dimethylformamide–pyridine. The final residue is dissolved in a mixture of dimethylformamide (5 ml) and pyridine (10 ml), and dimethoxytrityl chloride (5 g) is added. The whole mixture is shaken at room temperature for 4 days, diluted with triethylamine (2·5 ml) and 30% aqueous pyridine (20 ml), the shaking continued for 15 min, and the solution washed with two 10 ml portions of ether. The aqueous layer is diluted with 30% aqueous pyridine (10 ml) and extracted with chloroform (40 ml). The remaining aqueous layer is diluted with 30% aqueous pyridine again and extracted with chloroform. The chloroform extracts are combined, dried over anhydrous magnesium sulfate, treated with triethylamine (3 ml), and evaporated at 15°C : : 1 Torr. The residue is dissolved in a mixture of pyridine (10 ml) and triethylamine (2 ml), and the resulting solution is added dropwise with stirring into ether (1000 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure to yield 1·467 g (54%) of the triethylammonium salt of compound *Ib*. For $C_{29}H_{40}N_2O_6P$ (543·6) calculated: 64·15% C, 7·36% H, 5·15% N, 6·44% P; found: 63·84% C, 7·30% H, 4·88% N, 5·98% P. $R_F$ value: 0·25.

$P^1$-(Guanosine-5')-$P^2$-(2-aminoethyl-1) Pyrophosphate (*IIb*)

To a solution of compound *Ib* (218 mg; 0·4 mmol) in dimethylformamide (4 ml) there is added carbonyldiimidazole (320 mg) and the mixture is kept at room temperature for 2 days. The $R_F$ value in $S_1$ is changed from 0·25 (compound *Ib*) to 0·90 (compound *Ic*). Methanol (0·15 ml) is added, the mixture kept at room temperature for 15 min, evaporated (20°C/1 Torr), the residue dissolved in dimethyl sulfoxide (10 ml), and added to a dry cetyltrimethylammonium salt of guanosine 5'-phosphate (0·2 mmol) previously prepared as follows: guanosine 5'-phosphate is dissolved in 0·1M cetyltrimethylammonium hydroxide, the solution evaporated under occasional additions of ethanol, and the residue repeatedly coevaporated with toluene. The resulting dimethyl sulfoxide solution is concentrated (20°C/0·1 Torr) to the consistence of a sirup. After 4 days at room temperature, the sirup is diluted with the solvent system $S_1$ (5 ml) and chromatographed on a 40 × 20 × 0·6 cm layer of loose silica gel in $S_1$. The UV-absorbing and dimethoxytrityl-positive band ($R_F$ 0·60) is eluted with methanol, the eluate evaporated, and the residue dissolved in 50% aqueous acetic acid (5 ml). After 30 min, the mixture is applied to 1 sheet of paper Whatman No 3 MM and chromatographed in the solvent system $S_1$ for 5 days. The UV-absorbing band (10 cm from the start line) is eluted with 1% aqueous ammonia and the eluate is freeze-dried to afford 40 mg of the ammonium salt of compound *IIb*. For $C_{12}H_{19}N_6O_{11}\cdot P_2.2\,NH_3$ (519·30) calculated: 11·94% P; found: 11·51% P. Molecular weight, found: 536 (from the extinction at 260 nm). $R_F$ value: 0·02; $E_{Up}$ value: 0·50. Incubation of *IIb* with snake venom diesterase (pH 8) afforded guanosine 5'-phosphate.

6-Aminohexyl-1-phosphate (*IIIa*) (Barium Salt)

6-Amino-1-hexanol (8 g) is added to a cold mixture of phosphoric acid (32 g) and phosphorus pentoxide (25 g), the stirred mixture heated at 65°C for 2 h, diluted with water (300 ml), the whole heated at 100°C for 30 min, and neutralised with hot saturated aqueous barium hydroxide to the pink colour of the phenolphthalein paper. The barium phosphate is filtered off through a Celite layer and washed with hot water (1000 ml). The filtrate and washings are combined and concentrated under diminished pressure to the volume of about 100 ml. The concentrate is diluted with ethanol (200 ml), the precipitate collected with suction, and washed with ethanol and then ether to yield 6·2 g of the barium salt of compound *IIIa*. For $C_6H_{14}BaNO_4P$ (332·5) calculated: 4·22% N, 9·34% P; found: 4·25% N, 9·54% P, $R_F$ (paper): 0·12.

6-(Dimethoxytritylamino)hexyl-1-phosphate (*IIIb*)

A suspension of the barium salt of compound *IIIa* (3·32 g) and pyridinium Dowex 50 ion exchange resin (20 ml) in 50% aqueous pyridine is stirred until the salt passes into solution. The whole is then applied to a column (20 ml) of the same ion exchange resin. The column is eluted with 50% aqueous pyridine (100 ml), the eluate concentrated to the volume of about 20 ml (at 20°C/1 Torr), and the concentrate treated with tributylamine (5 ml) and pyridine (100 ml). The mixture is evaporated, the residue coevaporated with three portions of pyridine, the final residue dissolved in pyridine (20 ml), and the solution shaken with dimethoxytrityl chloride (7 g). The resulting homogeneous mixture is kept at room temperature for 7 days, diluted with triethylamine (5 ml) and 30% aqueous pyridine (50 ml), kept for additional 10 min, and washed with two 150 ml portions of ether. The aqueous phase is diluted with 30% aqueous pyridine (50 ml) and extracted with chloroform (50 ml); both these operations are repeated two times. The chloroform extracts are combined, dried over anhydrous magnesium sulfate, and evaporated first at 30°C/15 Torr and then at 20°C/1 Torr. The residue is dissolved in a mixture of pyridine (20 ml) and triethylamine

(2 ml) and the resulting solution is added dropwise into ether (1000 ml) to deposit a sirupous substance which is dissolved in pyridine (50 ml). The solution is evaporated at 20°C/1 Torr and the residue is coevaporated with two portions of toluene and once with chloroform to afford 3·6 g of the triethylammonium salt of compound *IIIb* as a solid foam. $R_F$ value: 0·21.

6-(Dimethoxytritylamino)hexyl-1-phosphoimidazolidate (*IIIc*)

To a solution of the triethylammonium salt of compound *IIIb* (140 mg; 0·2 mmol) in dimethylformamide (5 ml) there is added carbonyldiimidazole (162 mg), the mixture kept at room temperature for 2 days, treated with methanol (0·07 ml), kept for additional 15 min, and evaporated under diminished pressure. The residue ($R_F$ 0·71) is dissolved in dimethyl sulfoxide (5 ml) and the solution is used in the subsequent preparation.

Reaction of the Imidazolidate *IIIc* with t-RNA

A solution of cetyltrimethylammonium bromide (140 mg) in water (30 ml) is cooled down to 0°C and added at the same temperature to a solution of t-RNA (yeast) sodium salt (120 mg) in 30 ml of 0·005M Tris-HCl (pH 7·5). The mixture is kept at 0°C for 15 min, the precipitate collected by centrifugation, and washed at 0°C with four 40 ml portions of water. A suspension of the precipitate in water (10 ml) is then freeze-dried and the residue treated with the dimethyl sulfoxide solution of the imidazolidate *IIIc* from the preceding paragraph. The mixture is concentrated at 20°C/0·1 Torr to the volume of about 0·5 ml, the concentrate kept at room temperature for one week and treated with cold (0°C) ethanol (20 ml) and cold (0°) 3M sodium chloride (20 ml). After 15 min, the precipitate is collected by centrifugation, washed with three 20 ml portions of 1 : 1 ethanol — 3M-NaCl at 0°C, and stored at −20°C. After removal of the dimethoxytrityl group with 7M urea (pH 2·3) and separation of urea on DEAE-cellulose, the resulting substance coupled by 36% to the bromocyan-modified Sepharose. The renaturated preparation did not exhibit any acceptor activity[5].

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Translated by J. Pliml.