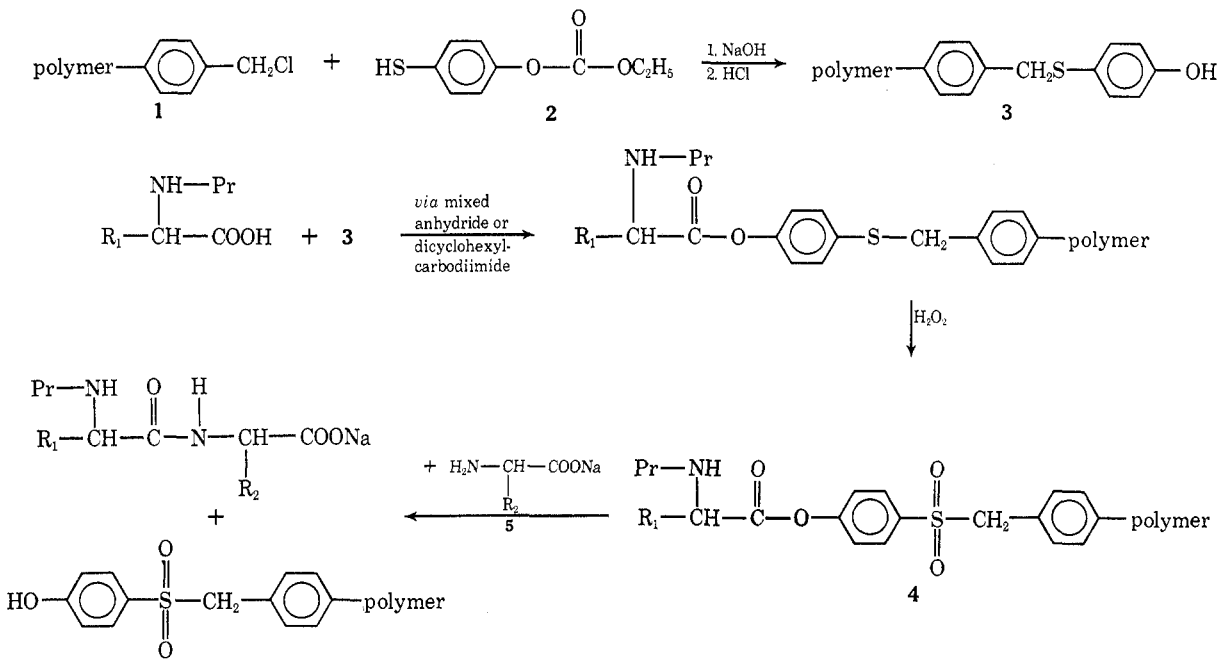
SCHEME I



Pr = protecting group

to 0.195 g (40% based on amount of glutamic acid attached to polymer). The product was purified by Sephadex G-15 chromatography using 50% acetic acid as solvent to give a ninhydrin-negative product which gave an amino acid analysis in agreement with the expected values. (amino acid ratios in acid hydrolyzate: Leu, 1.00; Glu, 1.06; Gly, 1.08.)

N-α-Carbobenzyloxycarbonyl-ω-nitro-L-arginyl-O-benzyl-L-seryl-L-valyl-L-γ-benzylglutamylglycine Ethyl Ester (Human Growth Hormone Sequence 180–184).—The general plan used for the previous tripeptide was used starting with the attachment of the penultimate C-terminal residue (N-*t*-BOC-L-glutamic acid γ-benzyl ester) to the support and followed by successive addition of the remaining residues. Oxidation with H_2O_2 converted the resin into an active, insoluble ester which, when treated with ethyl glycinate (free base), resulted in the formation of the desired protected pentapeptide removed from the polymer support. Chromatography on Sephadex G-25 using 50% acetic acid as solvent gave a product with the following amino acid analysis: Arg, 0.94; Ser, 1.00; Val, 1.10; Glu, 0.96; Gly, 1.06.

N-Benzoyl-L-leucylglycine Ethyl Ester.—N-*t*-BOC-L-leucine was coupled to the support with dicyclohexylcarbodiimide and the BOC group was removed by exposure to 50% trifluoroacetic acid–methylene chloride for 30 min. The amino group was neutralized with triethylamine, washed with DMF, and benzoylated by treatment with benzoyl chloride–pyridine in DMF at 5°. The benzoylated leucyl polymer was oxidized with H_2O_2 in acetic acid to give the active, insoluble ester. The resin was suspended in DMF and stirred for 24 hr with ethyl glycinate (free base). The dipeptide product was obtained by filtration and evaporation of the DMF. Crystallization of the residue from ethyl acetate–petroleum ether gave 0.18 g of product (38% yield based on amount of leucine attached to the polymer) whose optical activity and melting point agreed well with the reported values for the L isomer, $[\alpha]$ −34.1° (*c* 0.94, EtOH) (lit.[6] $[\alpha]$ −34.0°), mp 155–157° (lit.[6] mp 156–157°).

Registry No.—N-Benzoylglycine, 495-69-2; N-*p*-nitrobenzyloxycarbonyl-L-leucyl-L-γ-benzylglutamylglycine, 23025-41-4; N-α-carbobenzyloxycarbonyl-ω-nitro-L-arginyl-O-benzyl-L-seryl-L-valyl-L-γ-benzylglutamylglycine ethyl ester, 23025-42-5; N-benzoyl-L-leucylglycine ethyl ester, 2418-77-1.

Synthesis of a Diribonucleoside Monophosphate by the β-Cyanoethyl Phosphotriester Method[1]

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Since the β-cyanoethyl phosphotriester technique has proved useful for the synthesis of short-strand oligodeoxyribonucleotides in quantity,[1] it was of interest to see whether the technique could be extended to the synthesis of oligoribonucleotides. The major problem in the transition to the ribo series appeared to center on the steric effect of the substituent at the 2′ position. Specifically, would the condensation leading to a triester proceed satisfactorily with bulky substituents at the 2′ positions of the nucleosides? To answer this question a synthesis of uridylyl(3′-5′)-uridine *via* the β-cyanoethyl phosphotriester was attempted.

Nucleosides protected at the 2′-O position and at the 2′-O and 5′-O positions were prepared by utilizing the

(1) Part XVI in series on Nucleotide Chemistry. Part XV: R. L. Letsinger, K. K. Ogilvie, and P. S. Miller, *J. Amer. Chem. Soc.*, **91**, 3360 (1969). This research was supported by the Division of General Medical Sciences, National Institutes of Health (GM 10265).

acetylation technique of Fromageot, *et al.*,[2] and the ethoxyethylation reaction of Smrt and Chladek.[3] Thus treatment of 3′,5′-di-O-acetyluridine[2] with ethyl vinyl ether and trifluoroacetic acid in dimethylformamide at 0°, followed by hydrolysis with ammonium hydroxide, afforded 2′-O-(1-ethoxyethyl)uridine in 70% yield. 2′-O,5′-O-Di(1-ethoxyethyl)uridine was similarly prepared in 84% yield from 3′-O-acetyluridine.

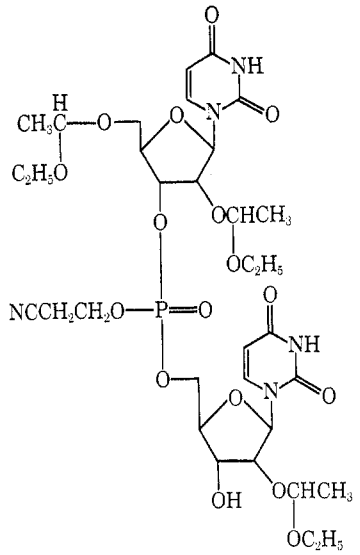
When 2′,5′-di-O-(1-ethoxyethyl)uridine was treated with a mixture of pyridinium β-cyanoethyl phosphate and 2,4,6-triisopropylbenzenesulfonyl chloride for 16 hr, β-cyanoethyl 2′,5′-di-O-(1-ethoxyethyl)uridine-3′ phosphate was produced. The yield, determined spectrophotometrically, was 92%. On removal of the protecting groups, uridine-3′ phosphate was obtained as the sole product. The high yield of the phosphorylated derivative shows that the bulky 2′-O protecting group does not impede reaction of the 3′-hydroxyl function with activated β-cyanoethyl phosphate.

Condensation of 2′-O-(1-ethoxyethyl)uridine with β-cyanoethyl 2′-O,5′-O-di(1-ethoxyethyl)uridine-3′ phosphate gave the β-cyanoethyl ester of 2′,5′-di-O-(1-ethoxyethyl)uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine (I). Removal of the protecting groups from a portion of this material showed a 54% conversion of 2′-O,5′-O-(1-ethoxyethyl)uridine to the dinucleoside phosphate derivative. Only one other product, which corresponded to uridine, was detected on the chromatograms.

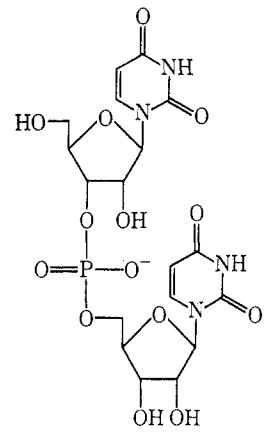
The major portion of the product mixture was separated by chromatography on silica gel. Elution with 50% tetrahydrofuran in ethyl acetate afforded 71 mg (33%) of the β-cyanoethyl ester of 2′,5′-di-O-(1-ethoxyethyl)uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine as a white solid, mp 77–80°. Treatment with ammonium hydroxide to remove the β-cyanoethyl groups followed by reaction with 5% aqueous acetic acid to remove the 1-ethoxyethyl groups gave uridylyl-(3′-5′)-uridine, which appeared as the sole uv-absorbing band on paper chromatography. That the linkage was 3′-5′ was shown by the fact that the UpU was completely degraded to uridine phosphate and uridine by ribonuclease, by spleen phosphodiesterase, and by snake venom phosphodiesterase.

It may therefore be concluded that the group at the 2′ position of uridine does not seriously interfere with the formation of the 3′-5′ phosphotriester link, and that the triester approach is applicable in the ribonucleotide series. Indeed, the yields in the condensation steps are close to those obtained in syntheses with deoxyribonucleosides. Since none of the 3′-3′ isomer of UpU was detected, it appears that the substituent at the 2′ position does inhibit formation of 3′-3′ phosphotriester links. This feature is advantageous, since it means that the nucleoside used in the condensation with the β-cyanoethyl phosphodiester derivative need not be protected at the 3′-OH.

When an attempt was made to isolate the β-cyanoethyl phosphotriester after treating I with 0.01 *N* hydrochloride acid to remove the 1-ethoxyethyl protecting groups, decomposition of the triester was observed. The products were uridylyluridine, uridine cyclic phosphate, and uridine. The lability of the ribonucleoside phosphotriester in acidic media no doubt stems from the neighboring 2′-hydroxyl group, which is well positioned to attack the phosphorus atom.



I



UpU

While the ethoxylethyl group served as a blocking group in this synthesis, it was found to be somewhat too sensitive to be an ideal protecting group. Some of the ethoxyethyl groups were lost in the course of isolating I. Thus, in addition to I, which was eluted from a silica gel column with 50% tetrahydrofuran–ethyl acetate, three additional products were obtained by subsequent elution of the silica gel column with tetrahydrofuran and methanol. These corresponded to substances derived from I by loss of one or more ethoxyethyl groups by hydrolysis. Each gave uridylyl-(3′-5′)-uridine on treatment with ammonium hydroxide follow by 5% aqueous acetic acid. The total amount of this material corresponded to 16% of the 2′,5′-di-O-(1-ethoxyethyl)uridine used in the synthesis.

Experimental Section

Ultraviolet spectra were obtained with a Beckman DU spectrophotometer. Infrared spectra were obtained with a Baird Model AB2 spectrometer with the sample in a potassium bromide disk unless otherwise specified. Elemental analyses were performed by H. Beck, Northwestern University, Evanston, Ill. Descending paper chromatography was carried out on Whatman 3MM paper with solvent A (isopropyl alcohol, concentrated ammonium hydroxide, and water, 7:1:2 by volume), solvent E (ethanol and 0.5 *M* ammonium acetate, 7:3 by volume and adjusted to pH 3.5 with glacial acetic acid), and solvent F (*n*-propyl alcohol, concentrated ammonium hydroxide, and water, 55:10:35 by volume). Electrophoretic separations were made on Whatman 3 MM paper strips with a Savant flat plate electrophoresis apparatus operated at 2000 V for 1 hr. Nucleosides and nucleotides were observed under ultraviolet light.

2′-O-(1-Ethoxyethyl)uridine.—3′,5′-Di-O-acetyluridine[2] (1.00 g) was dissolved in a mixture of dimethylformamide (2 ml) and ethyl vinyl ether (2 ml) and cooled in a Dry Ice–acetone bath. Trifluoroacetic acid (2 ml) was added; then the solution was successively warmed to 0° for 1 hr, cooled in a Dry Ice–acetone bath, neutralized with pyridine (10 ml), and poured into ice–water (200 ml). The mixture was extracted with chloroform (three 100-ml portions) and the chloroform extract was concentrated below 40°. Ethanol was added and the solution was evaporated. The residue was then taken up in concentrated ammonium hydroxide (100 ml). After 2 hr the ammonia was removed *in vacuo*. Thin layer chromatography on Eastman 6060

(2) H. P. Fromageot, B. E. Griffin, C. B. Reese, and J. E. Sulston, *Tetrahedron*, **23**, 2315 (1967).

(3) J. Smrt and S. Chladek, *Collect. Czech. Chem. Commun.*, **31**, 3800 (1966).

silica gel in ethyl acetate showed only one product (R_f 0.27). The oily residue was dissolved in a small amount of chloroform and applied to the top of a silica gel column (30 × 2 cm). After elution of the column with ethyl acetate (300 ml), 2′-O-(1-ethoxyethyl)uridine was eluted with 5% methanol in ethyl acetate and recrystallized from tetrahydrofuran–ether, mp 118–119°, yield 0.67 g (70%). Several additional recrystallizations afforded an analytical sample, mp 120–120.5°.

Anal. Calcd for $C_{13}H_{20}O_7N_2$: C, 49.36; H, 6.37; N, 8.86. Found: C, 49.76; H, 6.21; H, 8.86.

2′,5′-Di-O-(1-ethoxyethyl)uridine.—A solution containing 3′-O-acetyluridine[2] (2.22 g), dimethylformamide (30 ml), and ethyl vinyl ether (25 ml) was cooled to Dry Ice temperature and mixed with trifluoroacetic acid (7.5 ml). When the reaction was carried out and the product was isolated as described in the previous section, 2′,5′-di-O-(1-ethoxyethyl)uridine was isolated as an oil which would not crystallize. This oil was taken up in dry pyridine (25 ml) for use in the next step of the reaction sequence. Paper chromatography and uv analyses of the uridine obtained by acid hydrolysis showed that the pyridine solution was 0.26 *M* in 2′,5′-di-O-(1-ethoxyethyl)uridine.

Methanesulfonylation Test.—As a test for contamination by material with free 2′-OH, the uridine derivatives were subjected to the methanesulfonylation test of Fromageot, *et al.*[2]

Pure 2′-O and 3′-O monoprotected uridine derivatives lead to uncharged and cationic products, respectively.[2] Table I summarizes electrophoretic data for the 1-ethoxyethyl deivatives of uridine. Data are also presented for 3′-O-acetyluridine and 3′-O,5′-O-diacetyluridine, which were carried through the methanesulfonylation test as representatives of derivatives with a free 2′-OH. As shown in Table I, each of the ethoxyethyluridine derivatives yielded a single product and the product was uncharged. This is good evidence that each indeed had a blocking group at the 2′-O position, as expected from the mode of synthesis.

TABLE I

ELECTROPHORETIC MOBILITIES OF THE METHANESULFONYLATION PRODUCTS

Uridine derivative	R_m[a] of methanesulfonylation product
3′-O-Acetyluridine	−0.39
3′-O,5′-O-Diacetyluridine	−0.25
2′-O-(1-Ethoxyethyl)uridine	0.31
2′-O,5′-O-Di(1-ethoxyethyl)uridine	0.18

[a] Mobilities in 0.02 *M* sodium borate relative to uridine (R_m 1.00).

β-Cyanoethyl Ester of 2′,5′-Di-O-(1-ethoxyethyl)-urdine-3′ Phosphate.—A mixture of 2′-O,5′-O-di(1-ethoxyethyl)uridine (1 ml of 0.26 *M* solution in pyridine) and the pyridinium salt of mono-β-cyanoethyl phosphate (0.6 mmol), dried by stripping pyridine from it, was stirred with 2,4,6-triisopropylbenzenesulfonyl chloride (365 mg) in pyridine (1.0 ml) for 16 hr. Water (0.5 ml) was added with cooling and the aqueous solution was stirred for 24 hr to break up pyrophosphates. A saturated solution of aqueous tetraethylammonium bromide (0.5 ml) was added, and the mixture was extracted with chloroform (three 1-ml portions). The organic layer was separated and concentrated. Thin layer chromatography on Eastman cellulose, 6065, in solvent A showed only one spot, R_f 0.76. The optical density of an aliquot measured in water at 261 nm indicated a yield of the 2-cyanoethyl ester of 2′-O,5′-O-di(1-ethoxyethyl)-uridine-3′ phosphate of 92%. Hydrolysis with concentrated ammonium hydroxide (16 hr) afforded uridine-3′ phosphate (R_f 0.36 on paper with solvent F) as the sole uv-absorbing product.

β-Cyanoethyl Ester of 2′,5′-Di-O-(1-ethoxyethyl)uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine (I).—A mixture of 2′,5′-di-O-(1-ethoxyethyl)uridine (0.26 mmol) and the pyridinium salt of β-cyanoethyl phosphate (0.27 mmol), dried by stripping pyridine from it, was stirred with 2,4,6-triisopropylbenzenesulfonyl chloride (154 mg) in pyridine (0.5 ml) for 7 hr. 2′-O-(1-Ethoxyethyl)uridine (156 mg) in anhydrous pyridine (0.5 ml) and additional 2,4,6-triisopropylbenzenesulfonyl chloride (151 mg) were added; then the solution was stirred at room temperature for 20 hr and diluted to 5 ml with anhydrous pyridine.

Hydrolysis of an aliquot (0.20 ml) with concentrated ammonium hydroxide for 10 min and with 5% aqueous acetic acid for 2 hr gave UpU (R_f 0.57 on paper in solvent E) in 54% yield (spectrophotometric) based on 2′,5′-di-O-(1-ethoxyethyl)uridine employed in the reaction.

The rest of the pyridine solution was poured into 10 ml of 1.0 *M* aqueous sodium acetate (pH 7.7) and extracted with chloroform (three 10-ml portions). The organic layer was washed with 10 ml of 1.0 *M* sodium acetate solution and concentrated below 30°. Ethanol was added and stripped to remove traces of pyridine; then the gummy residue was dissolved in chloroform, applied to a silica gel column (34 × 3 cm), and eluted with mixtures of tetrahydrofuran and ethyl acetate (increasing precentages of tetrahydrofuran). The desired product eluted in 50% tetrahydrofuran–ethyl acetate. This fraction was stripped of solvent and the residue was dissolved in a small amount of tetrahydrofuran. The insoluble material was filtered off and washed with a small amount of tetrahydrofuran. Concentration of the filtrate and dilution with hexane afforded the 2-cyanoethyl ester of 2′,5′-di-O-(1-ethoxyethyl)uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine. This fine powder was collected by centrifugation, washed with hexane, and dried in a vacuum desiccator, mp 77–80°. The yield of I was 79 mg (33%): uv $\lambda_{max}^{95\%\ ethanol}$ 260 nm (ε 18,900) and 214 (8100); $\lambda_{min}^{95\%\ ethanol}$ 228 nm (ε 6000); ir λ_{max}^{KBr} 2.92, 3.37, 4.44, 5.91, 6.84, 7.22, 7.89, and 9.20 μ, among other absorptions.

Anal. Calcd for $C_{33}H_{50}O_{17}N_5P$: C, 48.35; H, 6.15; N, 8.54. Found: C, 48.35; H, 6.22; N, 8.31.

Further elution of the silica gel column with tetrahydrofuran–methanol yielded additional nucleotidic material. It was dissolved in water and an aliquot was chromatographed on paper in solvent A. Five uv-absorbing bands were observed, with R_f values of 0.13, 0.31, 0.46, 0.64, and 0.88. Each band was cut out and eluted with 1% aqueous ammonium hydroxide, and the optical density of the resulting solutions was measured at 261 nm. Electrophoresis showed that bands with R_f 0.13, 0.31, and 0.46 were dinucleoside monophosphates (R_m 0.33–0.35). Hydrolysis of a sample from each band gave uridylyluridine (R_f^F 0.38). The total yield of uridylyluridine from these three derivatives of I was 16% based on the original amount of 2′,5′-di-O-(1-ethoxyethyl)uridine. This material probably originates from I by loss of the acid-labile protecting groups on silica gel during the chromatographic separation.

Basic Hydrolysis.—Compound I (0.5 mg) was dissolved in methanol (0.1 ml) and applied to Whatman 3 MM paper. On developing the chromatogram in solvent A, only one uv-absorbing band was observed, corresponding to 2′,5′-di-O-(1-ethoxyethyl)-uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine (R_f 0.58).

Compound I (1 mg) was treated with concentrated ammonium hydroxide (0.5 ml) for 30 min and applied to Whatman 3 MM paper. After electrophoretic separation only one uv-absorbing band was observed, corresponding to 2′,5′-di-O-(1-ethoxyethyl)-uridylyl-(3′-5′)-2′-O-(1-ethoxyethyl)uridine (R_m −0.32 relative to uridine-3′ phosphate in phosphate buffer at pH 7.0).

Acid Hydrolysis.—Compound I (1 mg) was treated with 0.01 *N* hydrochloric acid (0.2 ml) for 30 min. After electrophoretic separation of an aliquot in phosphate buffer (pH 7.0) on paper, three uv-absorbing bands were observed with R_m 0.51, 0.36, and 0.00 relative to uridine-3′ phosphate. On chromatography of another aliquot on paper with solvent A, three uv-absorbing bands, R_f 0.09, 0.25, and 0.38, appeared.

These results show that the 2-cyanoethyl ester of uridylyl-(3′-5′)-uridine is unstable to the conditions necessary for removal of the acid-labile 1-ethoxyethyl protecting groups. The products of the decomposition of the triester appear to be the dinucleoside monophosphate (R_f 0.09, R_m 0.36), uridine (R_f 0.38, R_m 0.00), and uridine-2′,3′ cyclic phosphate (R_f 0.25, R_m 0.51).

Enzymatic Hyrolysis.—Compound I (5 mg) was treated with concentrated ammonium hydroxide (0.5 ml) for 2 hr and then chromatographed on paper with solvent A. The band (R_f 0.60) was cut out, eluted with water, lyophilized, and treated with 0.2 ml of 5% aqueous acetic acid for 2 hr at room temperature. Neutralization with ammonium hydroxide and chromatography on paper with solvent F yielded UpU as the sole nucleotidic product. On elution with water and lyophilization it was obtained as a white powder. Enzymatic degradation of uridylyl-(3′-5′)-uridine with ribonuclease, spleen phosphodiesterase,[1] and snake venom phosphodiesterase[1] gave the following ratios of nucleotide to nucleoside case: ribonuclease, Up/U 1.08:1; spleen, Up/U 1.09:1; snake venom, pU/U 1.02:1.

Registry No.—I, 22979-26-6; 2′-O-(1-ethoxyethyl)-uridine, 22979-27-7.