Nucleoside S-Alkyl Phosphorothioates. IV.[1] Synthesis of Nucleoside Phosphorothioate Monoesters

Alan F. Cook

Contribution from the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received July 7, 1969*

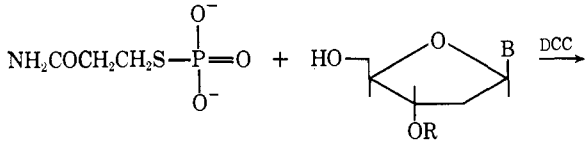
Abstract: A general synthesis of nucleoside phosphorothioates is reported. Nucleoside S-2-carbamoylethyl phosphorothioate derivatives were prepared from S-2-carbamoylethyl phosphorothioate and the appropriately protected nucleoside, using dicyclohexylcarbodiimide as the condensing agent. The nucleoside phosphorothioates were generated from their S-2-carbamoylethyl derivatives by treatment with alkali, and removal of base-stable protecting groups where necessary. In the same way, a phosphorothioate group has been introduced onto the 5′-hydroxyl group of a dinucleoside phosphate. Some chemical properties of nucleoside phosphorothioates and their derivatives have been investigated. In particular, the reactions of nucleoside phosphorothioates with halogen compounds have been used to prepare S-ethyl, S-2-cyanoethyl, and S-2-carbamoylethyl derivatives and dinucleoside phosphorothioates. The latter have been examined for their susceptibility to diesterases.

In recent years a number of nucleotide analogs have been prepared by modification of the sugar or base moiety, although modifications of the phosphate have been relatively few in number. Examples of the latter include nucleoside phosphites,[2] phosphonates,[3,4] and phosphorothioates.[5,6a,b] Nucleoside phosphorothioates provide an interesting series of compounds for study, since the substitution of one oxygen of the phosphate group by sulfur gives analogs containing a minimum of modification from the naturally occurring materials. The synthesis of this class of compounds cannot be accomplished with the usual condensing agents such as dicyclohexylcarbodiimide (DCC) unless the sulfur atom is protected, since the intermediates would otherwise undergo loss of sulfur.[7,8] Two approaches to the synthesis of nucleoside phosphorothioates have already been reported. The reaction between triimidazolyl-1-phosphine sulfide and partially protected nucleosides has been used to prepare 5′-phosphorothioates, although attempts to synthesize the 3′ isomers proved unsuccessful.[5] Adenosine 5′-phosphorothioate has been synthesized from a reaction between thiophosphoryl chloride and adenosine, using triethylphosphate as the solvent, and the 5′ isomer was obtained in good yield.[6] In contrast a reaction of 2′,3′-O-isopropylidene inosine with thiophosphoryl chloride gave, after removal of the isopropylidene group, a relatively poor yield of inosine 5′-phosphorothioate.[6b] We now wish to report a general synthesis of nucleoside phosphorothioates.

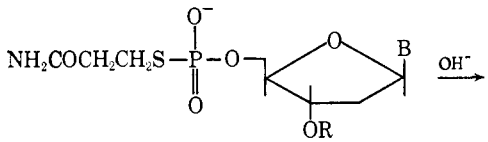
Previous papers in this series have dealt with nucleoside S-ethyl phosphorothioates,[9] and their use in oligonucleotide synthesis.[1] In this paper the sulfur atom of inorganic phosphorothioate has been protected by a 2-carbamoylethyl function, and the product condensed with a variety of partially protected nucleosides. After purification of the products, the alkali-labile 2-carbamoylethyl group has been removed to yield the corresponding nucleoside phosphorothioate. This approach is analogous to the method for nucleotide synthesis using 2-cyanoethyl phosphate.[10] Attempts to prepare S-2-cyanoethyl phosphorothioate were unsuccessful. The reaction of trilithium phosphorothioate with 3-bromopropiononitrile gave no material containing sulfur; a mercaptan-like odor was released, and trilithium phosphate was produced. In view of this result, attempts were made to protect the phosphorothioate molecule by reaction with other suitable halogen compounds. The reaction with 3-chloropropionamide proceeded quite smoothly, the progress of the reaction being followed by means of the silver nitrate test as described by Åkerfeldt.[11] After 24 hr the product was precipitated with ethanol, and the dilithium salt of S-2-carbamoylethyl phosphorothioate (**1**) was obtained in good yield. This material was converted into the pyridinium salt and used in a condensation with 3′-O-acetylthymidine (**2**, Scheme I), using DCC as the condensing agent. The products were separated by DEAE cellulose column chromatography, and 3′-O-acetylthymidine 5′-S-2-carbamoylethyl phosphorothioate (**3**) was obtained in 32% yield. The pyridinium salt of S-2-carbamoylethyl phosphorothioate was found to be almost insoluble in dry pyridine, and crystallized as needles from this solvent. Since this poor solubility may have accounted for the comparatively low yield of **3** from the condensation reaction, two condensations were carried out in which cosolvents were employed. The use of DMF as the cosolvent, in the presence of Dowex-50 resin (pyridinium form) increased the yield of **3** to 44%, and the use of hexamethylphosphoramide gave **3** in 63% yield. Hexamethylphosphoramide has therefore been routinely employed in this work as a cosolvent for condensation reactions. 3′-O-Acetylthymidylyl-(5′–5′)-thymidine 3′-O-acetate was isolated from these reactions in 5–10% yield, and was eluted from the ion-exchange column just before the desired product. This material was presumably formed from

(1) Paper III in this series: A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, **91**, 6479 (1969).
(2) A. Holý and F. Šorm, *Collect. Czech. Chem. Commun.*, **31**, 1562 (1966).
(3) G. H. Jones and J. G. Moffatt, *J. Amer. Chem. Soc.*, 90, 5337 (1968).
(4) A. Holý, *Tetrahedron Lett.*, 881 (1967).
(5) F. Eckstein, *J. Amer. Chem. Soc.*, **88**, 4292 (1966).
(6) (a) A. W. Murray and M. R. Atkinson, *Biochemistry*, 7, 4023 (1968); (b) A. Hampton, L. W. Brox, and M. Bayer, *ibid.*, 8, 2303 (1969).
(7) M. Mikolajczyk, *Chem. Ber.*, 99, 2083 (1966).
(8) F. Eckstein, *Tetrahedron Lett.*, 1157 (1967).
(9) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, **91**, 1522 (1969).
(10) G. M. Tener, *ibid.*, **83**, 159 (1961).
(11) S. Åkerfeldt, *Acta Chem. Scand.*, **16**, 1897 (1962).

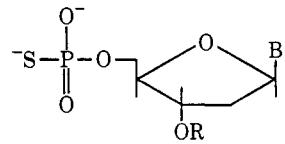
Scheme I. Synthesis of 5′-Phosphorothioates



1 2, R = $COCH_3$; B = thymine
5, R = $COCH_3$; B = 5-fluorouracil
8, R = 3′-O-acetylthymidine 5′-phosphoryl; B = thymine



3, R = $COCH_3$; B = thymine
6, R = $COCH_3$; B = 5-fluorouracil
9, R = 3′-O-acetylthymidine 5′-phosphoryl; B = thymine



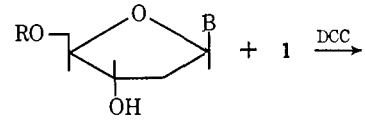
4, R = H; B = thymine
7, R = H; B = 5-fluorouracil
10, R = thymidine 5′-phosphoryl; B = thymine

inorganic phosphate, the latter being derived from breakdown of S-2-carbamoylethyl phosphorothioate. For the removal of the 2-carbamoylethyl group, compound **3** was treated with 0.2 *N* sodium hydroxide at 100° for 15 min. Thymidine 5′-phosphorothioate (**4**) was rapidly produced, and isolated in solid form as the barium salt. The over-all yield for the preparation of **4** from **2** was 52%. A similar sequence of reactions was used to prepare 5-fluorodeoxyuridine 5′-phosphorothioate. Thus 3′-O-acetyl-5-fluorodeoxyuridine (**5**) readily condensed with **1**, and the protected derivative **6** was obtained in the usual way. This material was not isolated in solid form, but instead treated directly with sodium hydroxide at 100° to give **7** in an over-all yield of 44%. This method was also used to introduce phosphorothioate onto the 5′-hydroxyl group of a dinucleoside phosphate. Thus, a condensation of **1** with thymidylyl-(3′–5′)-thymidine 3′-acetate (**8**) gave the carbamoylethyl derivative **9**, which was directly treated with alkali to give thymidylyl-(3′–5′)-thymidine 5′-phosphorothioate (**10**), a sulfur analog of the dinucleotide *d*-pTpT.

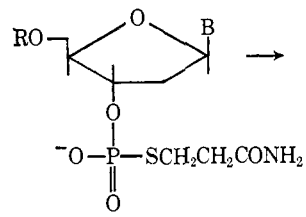
For the preparation of thymidine 3′-phosphorothioate, 5′-O-*p*-nitrobenzoylthymidine[12] (**11**, Scheme II) was used as the starting material. After condensation with **1** and column purification in the normal way, the fractions containing the S-carbamoylethyl derivative **12** were pooled, evaporated, and directly treated with alkali to give thymidine 3′-phosphorothioate (**13**) in an over-all yield of 56%. This yield is in fact higher than that obtained for the 5′-isomer, and thus the procedure provides an efficient method for the synthesis of 3′-phosphorothioates. The synthesis of deoxycytidine 3′-phosphorothioate was also accomplished in a similar manner. Condensation of N-anisoyl-5′-O-*p*-methoxytrityldeoxycytidine (**14**) with S-2-carbamoyl-

(12) K. E. Pfitzner and J. G. Moffatt, *J. Amer. Chem. Soc.*, 87, 5661 (1965).

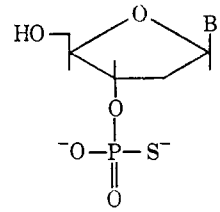
Scheme II. Synthesis of 3′-Phosphorothioates



11, R = NO_2—CO; B = thymine
14, R = MeO—C—$(C_6H_5)_2$; B = N_4-anisoyl-cytosine



12, R = NO_2—CO; B = thymine
15, R = MeO—C—$(C_6H_5)_2$; B = N_4-anisoyl-cytosine



13, B = thymine
16, B = cytosine

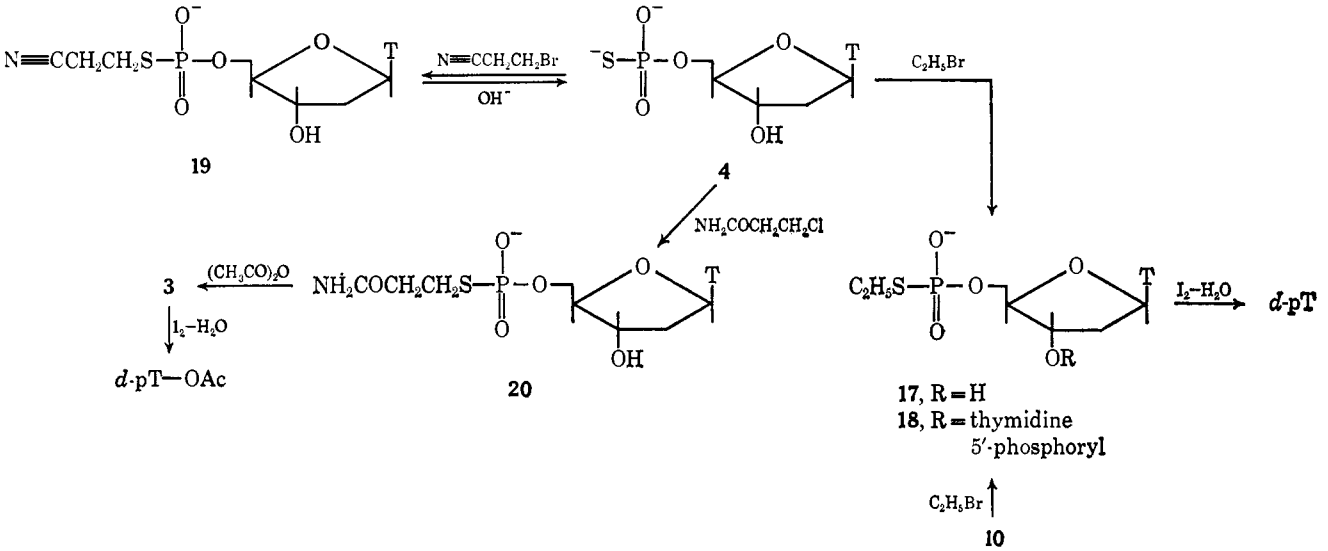
ethyl phosphorothioate in the usual way gave the fully protected deoxycytidine derivative **15**. Successive treatments with 80% acetic acid, concentrated ammonium hydroxide–methanol (1:1), and 0.2 *N* aqueous sodium hydroxide at 100° removed all the protecting groups to give deoxycytidine 3′-phosphorothioate (**16**) in 34% yield.[13]

Chemical Properties.

A predominant feature of these nucleoside phosphorothioates lies in the higher nucleophilicity of the phosphorothioate group as compared with its oxygen analog. This feature can be used in the preparation of a variety of derivatives by the reactions of the phosphorothioates with halogen compounds. Thus, reaction of thymidine 5′-phosphorothioate with ethyl bromide was complete after 24 hr at room temperature, and thymidine 5′-S-ethyl phosphorothioate (**17**, Scheme III) was obtained in 82% yield, and identified by chromatographic comparison with a sample prepared by another route.[9] The S-ethyl group of **17** was completely removed by treatment with aqueous iodine as previously described,[9] and thymidine 5′-phosphate was the sole product. This reaction sequence provides a method for the efficient conversion of **4** into its oxygen analog, and lends support to the designation of **17** as an S-ethyl rather than an O-ethyl derivative, since with aqueous iodine the latter would be expected to form a disul-

(13) Treatment with both ammonium hydroxide and sodium hydroxide was required since the former reagent did not completely remove the carbamoylethyl group after 16 hr, and it has previously been shown [H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, 83, 686 (1961)], that the N-anisoyl protecting group is relatively resistant to the action of sodium hydroxide.

Scheme III. Reactions of Thymidine 5′-Phosphorothioate (**4**)



19 **4** **3** **20** **17**, R = H **18**, R = thymidine 5′-phosphoryl **10**

fide (*vide infra* and ref 5) rather than undergo P–S cleavage. The absence of any detectable amounts of the O-ethyl isomer is in accordance with the known reactivity of phosphorothioates, the sulfur atom being the predominant nucleophilic center.[14] The reaction between ethyl bromide and the dinucleotide derivative **10** also proceeded to completion in 50% aqueous pyridine, and the corresponding S-ethyl derivative **18** was produced. This material was conveniently identified by paper chromatographic comparison with a sample which had been previously prepared by another route.[1] In the same way the reaction of **4** with 3-bromopropiononitrile for 16 hr at room temperature gave the S-2-cyanoethyl derivative **19** in 72% yield, which was isolated as the sodium salt. The reaction of **4** with 3-chloropropionamide was much slower than with the bromo compounds, but at 65° the reaction proceeded smoothly to give the S-2-carbamoylethyl derivative **20**. This material was identified by its conversion to **3** by acetic anhydride in pyridine.

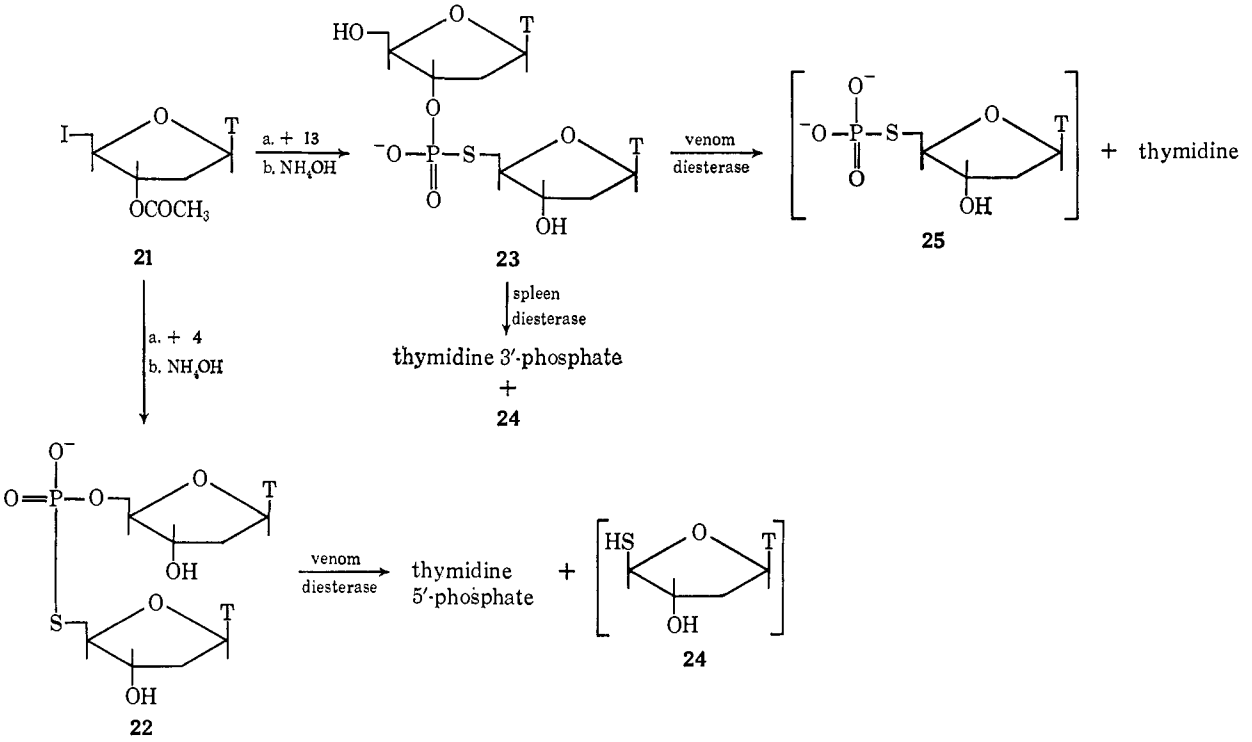
A rate study was made of the alkaline β elimination of thymidine 5′-S-2-cyanoethyl phosphorothioate (**19**) as compared with its oxygen analog.[15] These reactions were carried out in 0.1 *N* sodium hydroxide at room temperature, and examined by paper chromatography of aliquots, followed by elution and spectrophotometric assay of the appropriate spots from the chromatograms. The reaction with thymidine-2-cyanoethyl 5′-phosphate was complete in less than 20 min, with a half-life of approximately 4 min. This result is similar to that recorded for the 3′-isomer, its half-life in 0.1 *N* sodium hydroxide at 50° being reported as approximately 1 min.[10] In contrast, the sulfur analog **19** required 5 hr for complete reaction, with a half-time of 33 min. These results can be interpreted on the basis of phosphorothioate being a poorer leaving group than phosphate, the former being less stabilized due to less efficient π-electron overlap to form the P=S bond. The action of alkali on the S-2-carbamoylethyl compound **20** was also studied. The elimination reaction was much slower, and approximately half of the starting material still remained after treatment with 0.1 *N* sodium hydroxide at room temperature for 24 hr. Complete reaction occurred after treatment with 0.2 *N* sodium hydroxide at 100° for 10 min, however, with the production of thymidine 5′-phosphorothioate. The phosphorothioate moiety, therefore, can be protected by formation of an S-2-cyanoethyl derivative, and subsequently deprotected by treatment with aqueous alkali, and these reactions of protection and deprotection may be of use in a scheme for the synthesis of phosphorothioate analogs of oligonucleotides.

The reactions of thymidine phosphorothioates with a halonucleoside were also studied, with a view to the synthesis of dinucleoside phosphorothioates, which can be considered as dinucleoside phosphate analogs. The reaction between 3′-O-acetyl-5′-deoxy-5′-iodothymidine[16] (**21**, Scheme IV) and thymidine-5′-phosphorothioate proceeded readily at 70°, and after deacetylation of the product, the 5′,5′-dinucleoside phosphorothioate **22** was obtained, and isolated in solid form as the sodium salt. The reaction of thymidine 3′-phosphorothioate with **21** also proceeded smoothly and gave, after deacetylation, 3′,5′-dithymidine phosphorothioate (**23**). One plausible mechanism for the synthesis of these dinucleoside phosphorothioates **22** and **23** from the iodo derivative **21** involves initial formation of a 2,5′-anhydronucleoside, followed by attack of the phosphorothioate upon this intermediate. Accordingly, a study of the reaction of 3′-O-acetyl-2,5′-anhydrothymidine[17] with **4** was undertaken. After a reaction period of 7 hr at 70°, followed by deacetylation with ammonium hydroxide, significant amounts of **22** were detected; thus the participation of an anhydro intermediate cannot be ruled out.

These dinucleoside phosphate analogs provide interesting substrates for a study of the specificity of phosphodiesterases, since they contain a minimum amount of modification from the naturally occurring materials. Venom diesterase cleaved the 5′,5′-isomer **22** to give thymidine 5′-phosphate and a sulfur-containing material with paper chromatographic R_f close to thymidine (presumably 5′-deoxy-5′-mercaptothymidine **24**). This mode of cleavage by the enzyme is consistent with the observation by Razzell and Khorana[18] that a nucleo-

(14) S. Åkerfeldt, *Svensk Kem. Tidskr.*, 75, 231 (1963).
(15) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *J. Amer. Chem. Soc.*, 87, 2956 (1965).
(16) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).
(17) A. M. Michelson and A. R. Todd, *ibid.*, 816 (1955).
(18) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, 234, 2105 (1959).

Scheme IV. Synthesis and Enzymatic Cleavage of Dinucleoside Phosphorothioates



side 5′-phosphoryl group is the preferred moiety for binding to the enzyme. Venom diesterase cleaved the 3′,5′-isomer **23** at a slower rate than **22** or dithymidine 3′,5′-monophosphate, with the production of what is presumed to be **25** and thymidine; thus substitution of one oxygen atom of the phosphate group by sulfur reduces the rate of diester cleavage, although the binding of the sulfur analog to the enzyme must still occur to a significant extent. This extends the known substrate specificity of the enzyme. Although the 5′,5′-isomer **22** was resistant to spleen diesterase, the 3′,5′-isomer **23** was cleaved to thymidine 3′-phosphate and presumably **24**. These results are in contrast to those obtained from a dinucleoside phosphorothioate in which the sulfur atom was located in the thiophosphoryl function; in this case the material was resistant to both venom and spleen phosphodiesterase.[8] A poly-5′-thiouridylic acid preparation, however, was reported to be susceptible to the action of venom diesterase.[19]

The action of aqueous iodine upon the S-2-carbamoylethyl derivative **3** was investigated in order to determine whether the P–S bond could be cleaved in the same way as for the corresponding S-ethyl compound. Not surprisingly, this was found to be the case; the starting material was completely consumed after treatment overnight with an excess of reagent, and 3′-O-acetylthymidine 5′-phosphate was produced in high yield, together with a small amount of deacetylated material. In contrast, the action of iodine upon the monoester **4** did not produce P–S bond cleavage, but instead yielded a less polar, sulfur-containing product which was presumably the disulfide of **4** as previously reported.[5]

The self-condensation of the S-2-cyanoethyl derivative **19** was attempted with a view to the synthesis of oligonucleotide triesters containing S-2-cyanoethyl groups, which might yield a series of oligonucleotide phosphorothioates[20] upon treatment with alkali. This self-condensation was unsuccessful, since no oligomeric materials could be isolated from the products.

Experimental Section[21]

Dilithium S-2-Carbamoylethyl Phosphorothioate (1). A solution of trilithium phosphorothioate[11] (13.2 g) in water (150 ml) was treated with 3-chloropropionamide (16.1 g) in DMF (30 ml) for 24 hr at room temperature. The solution was filtered, and ethanol was added with stirring to the filtrate. The white precipitate was filtered off, washed with ethanol, and dried *in vacuo* to give the lithium salt of **1**, 14.6 g (74%).

Anal. Calcd for $C_3H_6Li_2NO_4PS$: C, 18.09; H, 3.05; P, 15.72; S, 16.27. Found: C, 18.02; H, 2.99; P, 15.70; S, 16.04.

3′-O-Acetylthymidine 5′-S-2-Carbamoylethyl Phosphorothioate (3). The dilithium salt of **1** (1.18 g) was converted into the pyridinium salt by passage through a column of Dowex-50 resin (pyridinium form). The eluate was evaporated to dryness, 3′-O-acetylthymidine (568 mg, 2 mmol) was added to the residue, and the mixture was dried by repeated evaporation of added portions of dry pyridine. Hexamethylphosphoramide (5 ml), pyridine (5 ml), and DCC (2.06 g) were added, and the mixture was shaken for 4 days. The solids were removed by filtration and washed with dry pyridine, and the filtrate and washings were treated with water (10 ml) overnight at 0°. The precipitate was filtered off, and the filtrate was evaporated to low volume. Addition of water (25 ml) yielded a gummy precipitate which was discarded, since it did not contain any ultraviolet-absorbing material. The solution was adjusted to pH 7 and applied to a DEAE cellulose column (45 × 3.5 cm, acetate form) which was eluted with a linear gradient of 2 l. of triethylammonium acetate, pH 6 (0.005 *M*) in the mixing vessel and 2 l. of the same buffer (0.05 *M*) in the reservoir. The fractions which were eluted from the column at a buffer strength of 0.03–0.05 *M* were pooled and evaporated to dryness, and the residue was converted into the sodium salt by passage through a column of Dowex-50 resin (sodium form). The column eluate was evaporated to dryness, and the residue was dried and dissolved in dry methanol (3 ml). Addition of dry ether (150 ml) gave a precipitate which was collected by centrifugation, washed with ether (three 10-ml portions), and dried *in vacuo* to give 596 mg (63%) of **3** as the sodium salt, $\lambda_{max}^{H_2O}$ 265 mμ (ε 9000).

(19) A. M. Michelson, *J. Chem. Soc.*, 979 (1962).

(20) Polyribonucleotides containing this kind of phosphorothioate backbone have recently been prepared by enzymatic methods; F. Eckstein and H. Gindl, *F.E.B.S. Lett.*, **2**, 262 (1969).

(21) The general procedures were carried out as previously described in ref 9. Dry solvents were employed for all condensation reactions using DCC. Fractions of 20 ml were always collected from ion exchange cellulose columns.

Anal. Calcd for $C_{15}H_{21}N_3NaO_9PS$: C, 38.06; H, 4.47; P, 6.54; S, 6.77. Found: C, 37.84; H, 4.89; P, 6.60; S, 6.93.

3′-O-Acetylthymidylyl-(5′–5′)-thymidine 3′-O-acetate, 1000 OD_{267} units (5%), was eluted from the column just before **3**, and was identified by paper chromatographic comparison with a sample which had been previously prepared.[9]

Thymidine 5′-Phosphorothioate (4). This procedure was the same as described for **3**, except that the pooled fractions from the DEAE cellulose column were evaporated to dryness and directly treated with aqueous sodium hydroxide (0.2 *N*, 20 ml) at 100° for 10 min. The product was neutralized by addition of Dowex-50 resin (pyridinium form), and the resin was filtered off and washed with water. The filtrate was adjusted to pH 7.5 and applied to a DEAE cellulose column (45 × 3.5 cm, bicarbonate form), which was eluted with a linear gradient of 2 l. of triethylammonium bicarbonate, pH 7.5 (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.2 *M*) in the reservoir. The material which emerged at 0.1 *M* buffer strength was evaporated, and the residue was dissolved in water and passed through a column of Dowex 50 resin (hydrogen form). The eluate was adjusted to pH 7 with barium hydroxide, and evaporated to 10 ml. The precipitate was removed by centrifugation and discarded, and ethanol (20 ml) was added to the filtrate. The flocculent white precipitate was collected by centrifugation, washed with acetone (two 10-ml portions), and ether (one 10-ml portion), and dried *in vacuo* to give 533 mg (52%) of **4** as the dihydrated barium salt, $\lambda_{max}^{H_2O}$ 265 mμ (ε 9470).

Anal. Calcd for $C_{10}H_{13}BaN_2O_7PS \cdot 2H_2O$: C, 23.57; H, 3.36; N, 5.50; S, 6.29. Found: C, 23.65; H, 3.44; N, 5.55; S, 6.36.

5-Fluorodeoxyuridine 5′-Phosphorothioate (7). A solution of 3′-O-acetyl-5-fluorodeoxyuridine (**5**, 1.15 g, 4 mmol) and pyridinium S-2-carbamoylethyl phosphorothioate (**1**, 12 mmol) in pyridine was dried as previously described. Pyridine (10 ml), hexamethylphosphoramide (10 ml), and DCC (4 g) were added, and the mixture was shaken for 4 days. The product **6** was purified as described for **3**, except that the DEAE column (60 × 4.7 cm) was eluted with a gradient of 4 l. of acetate buffer, pH 6 (0.005 *M*) in the mixing vessel, and 4 l. of the same buffer (0.2 *M*) in the reservoir. The fractions which emerged at 0.04–0.06 *M* buffer strength were pooled and evaporated, and the residue was treated with sodium hydroxide (0.2 *N*) at 100° for 10 min and neutralized in the usual way. The product was purified by DEAE cellulose column (60 × 4.7 cm, bicarbonate form) chromatography, using a gradient of 4 l. of bicarbonate buffer, pH 7.5 (0.005 *M*) in the mixing vessel, and 4 l. of the same buffer (0.2 *M*) in the reservoir. The compound was obtained from fractions 290–400, and isolated in the barium form as described for **4**, to give 1.04 g (49%) of **7** as the dihydrate; $\lambda_{max}^{H_2O}$ 267 mμ (ε 8330).

Anal. Calcd for $C_9H_{10}BaFN_2O_7PS \cdot 2H_2O$: C, 21.05; H, 2.74; N, 5.45; S, 6.24. Found: C, 21.16; H, 2.64; N, 5.32; S, 5.99.

Preparation of 10. The pyridinium salt of **8** (0.1 mmol) was treated with **1** (0.4 mmol) in pyridine (1 ml) and hexamethylphosphoramide (1 ml) for 3 days with shaking, using DCC (200 mg) as the condensing agent. The product was treated in the usual way, and purified on a DEAE column (30 × 1.7 cm, acetate form) using 2 l. of acetate buffer (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.25 *M*) in the reservoir.

Fractions 55–69 were pooled, evaporated, and treated with 0.2 *N* sodium hydroxide (10 ml) at 100° for 15 min. The product was eluted from a DEAE column (50 × 1.7 cm, bicarbonate form) using a gradient of 2 l. of bicarbonate buffer (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.25 *M*) in the reservoir. Fractions 102–112 were pooled to give 334 OD_{267} units (18%) of **10**; $\lambda_{max}^{H_2O}$ 267 mμ. This material was completely degraded to mononucleotides using venom phosphodiesterase.

Thymidine 3′-Phosphorothioate (13). A solution of 5′-O-*p*-nitrobenzoylthymidine[12] (782 mg, 2 mmol) and **1** (6 mmol, pyridinium salt) in pyridine (5 ml) and hexamethylphosphoramide (5 ml) was treated with DCC (2 g) for 4 days with shaking. After the usual treatment, the material was applied to a DEAE column, (50 × 4 cm, acetate form) which was eluted with a gradient of 4 l. of acetate buffer (0.005 *M*) in the mixing vessel, and 4 l. of 0.1 *M* buffer in the reservoir. Fractions 120–250 were combined, evaporated, treated with sodium hydroxide (0.2 *M*, 30 ml) in methanol (5 ml) at 100° for 10 min, and the product was purified as described for **4**, to give 548 mg (56%) of **13** as the monohydrated barium salt; $\lambda_{max}^{H_2O}$ 266 mμ (ε 9360).

Anal. Calcd for $C_{10}H_{13}BaN_2O_7PS \cdot H_2O$: C, 24.42; H, 3.05; S, 6.52. Found: C, 24.24; H, 3.26; S, 6.62.

Deoxycytidine 3′-Phosphorothioate (16). N-Anisoyl-5′-O-*p*-methoxytrityldeoxycytidine[22] (**14**, 0.5 mmol) and **1** (1.5 mmol) in pyridine (2.5 ml) and hexamethylphosphoramide (2.5 ml) were treated with DCC (1 g) for 3 days with shaking. After the usual water treatment and filtration procedure, the filtrate was evaporated to dryness, dissolved in 50% aqueous ethanol (60 ml), and applied to a DEAE cellulose column (30 × 1.8 cm, acetate form) which was eluted with a gradient of 2 l. of acetate buffer (0.005 *M*) in 50% ethanol in the mixing vessel, and 2 l. of 0.1 *M* buffer in 50% ethanol in the reservoir. Fractions 51–114 were pooled, evaporated, and treated successively with 80% acetic acid (20 ml) for 3 hr, concentrated ammonium hydroxide (10 ml) in methanol (15 ml) for 16 hr, and then aqueous sodium hydroxide (0.2 *N*, 15 ml) in methanol (15 ml) for 15 min at 100°. The solution was neutralized with Dowex-50 resin, and the filtrate was partially evaporated to remove methanol, and extracted with ether (three 20-ml portions). The aqueous layer was purified by column chromatography as described for **4**, to give 1565 OD_{280} units (34%) of deoxycytidine 3′-phosphorothioate. A sample was isolated as the barium salt, $\lambda_{max}^{0.1\ N\ HCl}$ 278 mμ (ε 12,360).

Anal. Calcd for $C_9H_{12}BaN_3O_6PS \cdot H_2O$: C, 22.68; H, 2.96; S, 6.73. Found: C, 22.61; H, 3.03; S, 6.43.

Reaction of 4 with Ethyl Bromide. A solution of **4** (102 mg, barium salt) in 50% aqueous pyridine (4 ml) was treated with ethyl bromide (0.1 ml) for 24 hr at room temperature. The solution was evaporated to dryness, dissolved in water (25 ml) and applied to a DEAE column (45 × 2.3 cm, bicarbonate form) which was eluted with 2 l. of bicarbonate buffer, pH 7.5 (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.15 *M*) in the reservoir. Fractions 55–69 were pooled, evaporated, converted into the sodium form, dissolved in dry methanol (3 ml), and precipitated by addition of ether (100 ml). The precipitate was collected by centrifugation, washed with ether (three 10-ml portions) and dried *in vacuo* to give 63 mg (82%) of **17**. This material was chromatographically identical with a sample prepared by a published procedure.[9]

Preparation of 19. A solution of **4** (70 mg, barium salt) in 50% aqueous pyridine (4 ml) was treated with 3-bromopropiononitrile (0.2 ml) overnight at room temperature. After column purification of the product as described for the S-ethyl derivative, fractions 41–50 were pooled and evaporated to dryness. The residue was dissolved in water (20 ml) and adsorbed onto Norit. After the carbon had been washed thoroughly with water, the nucleotidic material was recovered by elution with 10% aqueous pyridine (four 50-ml portions). The combined eluates were evaporated to dryness, and the residue was converted into the sodium form and precipitated from dry methanol (3 ml) by addition of ether (50 ml). The solid was collected by centrifugation, washed with ether (three 10-ml portions), and dried *in vacuo* to give 41 mg (71%) of **19**; $\lambda_{max}^{H_2O}$ 266 mμ (ε 8975).

Anal. Calcd for $C_{13}H_{17}N_3NaO_7PS$: C, 37.67; H, 4.15; S, 7.76. Found: C, 37.46; H, 4.90; S, 7.96.

Reaction of 4 with 3-Chloropropionamide. A solution of **4** (60 mg, barium salt) in 50% aqueous pyridine (2 ml) was heated with 3-chloropropionamide (180 mg) at 65° for 17 hr. The product was purified by the procedure described for **19**, to give **20**, 38 mg (75%) as the sodium salt, $\lambda_{max}^{H_2O}$ 264 mμ (ε 8520).

Anal. Calcd for $C_{13}H_{19}N_3NaO_8PS$: N, 9.74; S, 7.43. Found: N, 9.89; S, 7.58.

A sample (1 mg) of this material was converted into the pyridinium form and treated with acetic anhydride (0.25 ml) in pyridine (0.5 ml) for 17 hr. Water (0.5 ml) was added to the product, and after 1 hr the solution was evaporated to dryness. The residue was found to be chromatographically identical with **3**.

Synthesis of 22. Thymidine 5′-phosphorothioate (77 mg) in 50% aqueous pyridine (4 ml) was treated with 3′-O-acetyl-5′-deoxy-5′-iodothymidine (**21**, 385 mg) at 70° for 7 hr. The product was treated overnight with concentrated aqueous ammonia (4 ml), and the solvents were then removed by evaporation. Addition of water (25 ml) to the residue gave a white solid, which was removed by filtration and washed with water. The filtrate was adjusted to pH 7.5 and purified by passage through a DEAE cellulose column (60 × 2.3 cm, bicarbonate form). The column was eluted with a linear gradient of 2 l. of bicarbonate buffer, pH 7.5 (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.1 *M*) in the reservoir. Fractions 55–70 were pooled, evaporated to dryness, and **22**, 81 mg (78%) was obtained from the residue by conversion into the sodium form and precipitation from dry methanol (3 ml) by addition of ether (100 ml); $\lambda_{max}^{H_2O}$ 267 mμ (ε 17,260).

(22) This material was prepared by the same method as described for the corresponding N-benzoyl compound: H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3821 (1963).

Anal. Calcd for $C_{20}H_{26}N_4NaO_{11}PS \cdot 3H_2O$: P, 4.85; S, 5.02. Found: P, 4.84; S, 4.97.

Preparation of 23. A solution of **13** (70 mg, barium salt) and the iodonucleoside **21** (340 mg) in 50% aqueous pyridine (3 ml) was heated at 70° for 7 hr. The purification procedure as described for **22** was used to obtain 60 mg (85%) of **23**; $\lambda_{max}^{H_2O}$ 267 mμ (ε 17,880).

Anal. Calcd for $C_{20}H_{26}N_4NaO_{11}PS$: C, 41.08; H, 4.48; S, 5.48. Found: C, 40.94; H, 5.27; S, 5.29.

Treatment of 3 with Aqueous Iodine. A solution of **3** (50 mg) in 50% aqueous acetone (2 ml) was treated with iodine (100 mg) for 17 hr, and then diluted with water (10 ml) and extracted with ether (three 5-ml portions). The residual ether was removed from the aqueous layer by evaporation, and the solution was applied to a DEAE column (50 × 2.3 cm, acetate form) which was eluted with a gradient of 2 l. of acetate buffer, pH 6 (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.2 *M*) in the reservoir. 3′-O-Acetylthymidine 5′-phosphate, 731 OD_{267} units (77%) was eluted from the column in fractions 72–83, and was identified by comparison with an authentic sample.[23]

Acknowledgments. Thanks are extended to Dr. V. Toome for ultraviolet spectra, to Dr. F. J. Scheidl for microanalyses, and to Dr. A. L. Nussbaum for helpful discussions.

(23) P. T. Gilham and H. G. Khorana, *J. Amer. Chem. Soc.*, 80, 6212 (1958).

Synthesis of Cholecystokinin–Pancreozymin. I. The C-Terminal Dodecapeptide

Miguel A. Ondetti, Josip Pluščec, Emily F. Sabo, John T. Sheehan, and Nina Williams

Contribution from The Squibb Institute for Medical Research, New Brunswick, New Jersey 08903. *Received August 11, 1969*

Abstract: A dodecapeptide amide with the structure proposed by Mutt and Jorpes for the C-terminal sequence of the intestinal hormone cholecystokinin–pancreozymin has been synthesized by the condensation of three fragments: di-, tetra-, and hexapeptide. All the fragments were prepared by the stepwise procedure using active esters, and the coupling of the intermediate peptides was carried out by the azide method. The synthetic dodecapeptide (XX) was identical with the natural counterpart isolated by partial tryptic digestion of the hormone. This C-terminal sequence has all the biological properties of cholecystokinin–pancreozymin.

In 1928 Ivy and Oldberg[1] proposed the name cholecystokinin for the chemical entity present in the crude extracts of intestinal mucosa that was responsible for the contractile activity of these extracts on the gall bladder of the dog. Fifteen years later, Harper and Raper[2] named as pancreozymin the compound present in the same type of extracts and responsible for the stimulation of pancreatic enzyme release. The careful isolation carried out at the Karolinska Institute by Mutt and Jorpes[3] has shown that both biological activities are elicited by one compound that they have, at least temporarily, designated as cholecystokinin–pancreozymin (CCK-PZ). The structural studies have led to the conclusion that this hormone is a single-chain polypeptide of thirty-three amino acids. The partial sequence of Scheme I represents the latest published results of these investigations.[4] Through a personal communication from Professor J. E. Jorpes and Dr. V. Mutt we have learned that the specific sequence they have proposed for the C-terminal dodecapeptide is: Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp Phe-NH_2. This dodecapeptide can be isolated by partial tryptic digestion of CCK–PZ.[4]

Scheme I

Lys-(Ala_1, Gly_1, Pro_1, Ser_1)-Arg-Val-(Ile_1, Met_1, Ser_1)-Lys-Asn-(Asx_1, Glx_1, His_1, Leu_2, Pro_1, Ser_2)-Arg-Ile-(Asp_1, Ser_1)-Arg-Asp-(Gly_1, SO_3H | Met_2, Trp_1, Tyr_1)-Asp-Phe-NH_2

The most notable feature of this C-terminal sequence is its resemblance to the similar portion of the gastric hormone gastrin[5] and also to the decapeptide caerulein[6] (Scheme II) isolated by Erspamer and his coworkers from the skin of the amphibian *Hyla caerulea.* While the similarity to gastrin extends strictly up to the C-terminal pentapeptide, in the case of caerulein it extends up to the octapeptide, except for the replacement of methionine by threonine. The presence of a tyrosine residue with the phenolic hydroxyl esterified with sulfuric acid is another interesting feature of this partial sequence. In the case of gastrin, both the sulfated and the unsulfated forms have been isolated, but in the case of cholecystokinin and caerulein only the sulfated forms have so far been found.

Scheme II

Glu-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Glu-
SO_3H
|
Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2
porcine gastrin II

SO_3H
|
Glu-Gln-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH_2
caerulein

(1) A. C. Ivy and E. Oldberg, *Amer. Physiol.*, **86**, 599 (1928).
(2) H. A. Harper and H. S. Raper, *J. Physiol.* (London), **102**, 115 (1943).
(3) J. E. Jorpes, V. Mutt, and K. Toczko, *Acta Chem. Scand.*, **18**, 2408 (1964).
(4) V. Mutt and J. E. Jorpes, *Eur. J. Biochem.*, **6**, 156 (1968).
(5) H. J. Tracy and R. A. Gregory, *Nature*, **204**, 935 (1964).
(6) A. Anastasi, V. Erspamer, and R. Endean, *Experientia*, **23**, 699 (1967).