[CONTRIBUTION FROM THE STERLING CHEMISTRY LABORATORY AND THE BINGHAM OCEANOGRAPHIC LABORATORY, YALE UNIVERSITY]

CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS. XXXII. THE NUCLEOSIDES OF SPONGES. I.[1]

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In the fall of 1945 the senior author collected in the shallow waters off Elliot Key, Florida, a number of sponges of a species which had not previously been described. Representatives of the same species were found in relative abundance in the waters surrounding Bimini Islands, Bahamas, in June, 1948 by the senior author and the eminent taxonomist of Porifera, Dr. M. W. de Laubenfels,[3] who has since described this species under the name *Cryptotethia crypta* (1). Within a few hours after their collection the Florida specimens had been preserved in a 5% formalin-seawater solution, and were dried later in a vacuum-oven at 60°. The Bimini sponges had been dried in the open air only. The two lots of sponges were investigated separately in 1949. Some of the results of this study have been the subject of a preliminary communication (2).

During the acetone extraction of the lipids of the Florida sponges in a modified Soxhlet apparatus (3) there separated from the boiling solvent a rather copious quantity of a nicely crystalline material in a yield corresponding to about 2% of the dry sponges. Upon recrystallization of this material from dilute ethanol and from water a product was obtained in the form of clear prisms, m.p. 246–247°. It was optically active, $[\alpha]_D$ +80.0°, it contained nitrogen, and it gave analytical data in agreement with the empirical formula $C_5H_7NO_3$. In an aqueous solution its absorption spectrum (Figure 1) showed a single maximum at 269 mμ which was independent of the *p*H of the solvent. The great similarity of this spectrum to that of thymine desoxyriboside (4) made it probable that the compound was a pyrimidine nucleoside of the formula $C_{10}H_{14}N_2O_6$.

As in certain other pyrimidine nucleosides (5) the fission of the glycosidic linkage was accomplished only under drastic conditions which led to the destruction of the carbohydrate moiety. The pyrimidine fragment, however, was readily identified as thymine, m.p. 321°.

The identification of thymine as one part of the nucleoside proved the other part, $C_5H_9O_4$, to be derived from a pentose. The presence in this fragment of three hydroxyl groups was shown by the facile formation of a tribenzoate and a tri-*p*-bromobenzoate. When titrated with periodic acid according to the pro-

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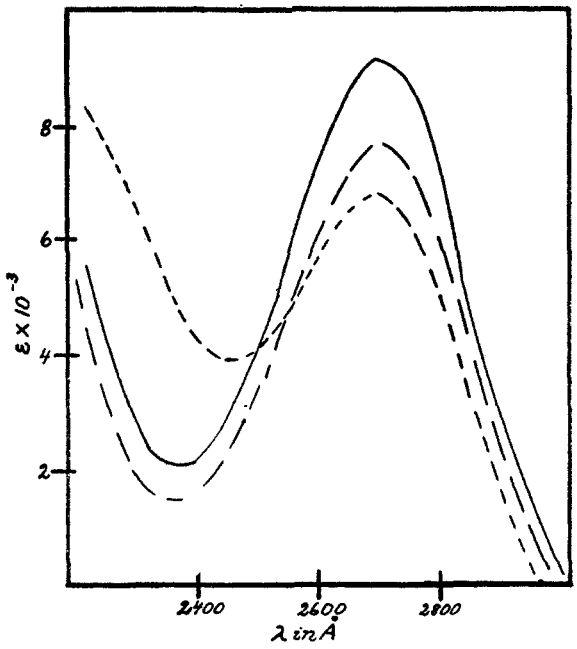
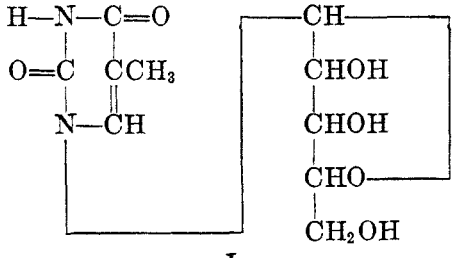


FIGURE 1. ABSORPTION SPECTRUM OF SPONGOTHYMIDINE. ———— in water; — — — in 0.1 *N* HCl; —— —— —— in 0.1 *N* NaOH.

cedure recommended by Lythgoe and Todd (6) the nucleoside consumed only one mole of the acid and this without formation of formic acid. The pentose residue is therefore present in the furanoside structure (I).



I

The evidence presented so far shows convincingly that the nucleoside is a pentofuranosylthymine. Since no compound of this composition has been previously described it is proposed to name it *spongothymidine*.

Great difficulties, as yet not overcome, were encountered in the attempts to isolate the pentose fragment of the nucleoside. They were to be anticipated on the basis of Levene's (7) experiences with the known D-ribosyl- and 2-desoxy-D-ribosyl-pyrimidines. Thus a hydrolysis of 2-desoxy-D-ribofuranosyl-thymine without the destruction of the carbohydrate has not yet been accomplished, and the nature of the latter has only been established indirectly. Similarly the

fission of uridine into uracil and D-ribose has not yet been possible. If, however, uridine is first hydrogenated catalytically to dihydrouridine, the latter may be readily hydrolyzed to afford D-ribose.

An analogous procedure was used in some of the numerous unsuccessful attempts to obtain the pentose from spongothymidine. Unfortunately, however, the nucleoside resisted catalytic hydrogenation. Even when treated with a variety of platinum catalysts under pressures of 400–500 pounds of hydrogen and at temperatures of 120° the material was recovered unchanged.

The attractive hypothesis that spongothymidine is identical with ribofuranosylthymine, which is as yet unknown, finds little if any support in the rotational data. The molecular rotation of uridine in 5% sodium hydroxide is +14° and that of spongothymidine in 8% sodium hydroxide is +206°. It does not seem probable that the introduction of a methyl group in the 5^1 position of uridine should be accompanied by so large a change in molecular rotation. It seems more likely that the high positive rotation of spongothymidine is caused by a pentose unit such as D-xylose which is more dextrorotatory than D-ribose.

Spongothymidine was accompanied by a small amount of a more soluble levorotatory product of m.p. 192–193°, $[\alpha]_D$ −42.5°, and the empirical formula $C_{11}H_{15}N_5O_5$. Since it is known (7) that among the well established nucleosides those derived from pyrimidines are dextrorotatory in contrast to the levorotatory purine derivatives, it appeared probable that this new nucleoside was a purine analogue of spongothymidine. This probability was strengthened by the ultraviolet absorption spectrum of the compound which showed, like those of other purine nucleosides, (4), a single maximum in water and 0.1 *N* sodium hydroxide and two maxima in 0.1 *N* hydrochloric acid (Figure 2).

In conformity with the behaviour of other purine nucleosides this levorotatory nucleoside was readily hydrolyzed to give a purine in a nicely crystalline form, m.p. 276°. Because of its tendency, common among purines, to form hydrates of various proportions, reproducible and informative analytical results were at first difficult to obtain. It was of interest, however, that all analyses gave the unexpected ratio of six carbon atoms to five nitrogen atoms, or one carbon atom more than is normally found among purines of animal origin. An anhydrous product was eventually obtained by refluxing the purine with xylene and removal of the water by co-distillation. Its analytical data were in agreement with the formula $C_6H_7N_5O$ and suggested that the compound is a methylaminoöxypurine. The presence of the amino group was definitely established by diazotization which gave the corresponding dioxycompound. The empirical formula of the latter, $C_6H_6N_4O_2$, also indicates the presence of a methyl group.

Lack of material has so far prevented the elucidation of the structure of the purine and the carbohydrate residue. When more material has become available the isolation of the latter should prove to be much easier than that of the corresponding spongothymidine residue. The present evidence, however, appears to be adequate to permit one to state with some certainty that this purine nucleo-

side is of a type not heretofore encountered in nature. It is proposed to refer to it as *spongosine* until such time as this trivial name can be replaced by a more detailed one.

Extraction of the Bahamas sponges also afforded a mixture of compounds difficultly soluble in boiling acetone. It did not however, consist primarily of nucleosides, but of free pyrimidine and purine bases with a preponderance of thymine and a small percentage of the purine mentioned above. The difference in the behavior upon extraction of the Florida and Bahamas sponges is probably due to the differences in treatment received by the sponges prior to their extraction, which have been outlined in the introduction. It seems reasonable to assume that in the Bahamas sponges the nucleosides underwent autolysis

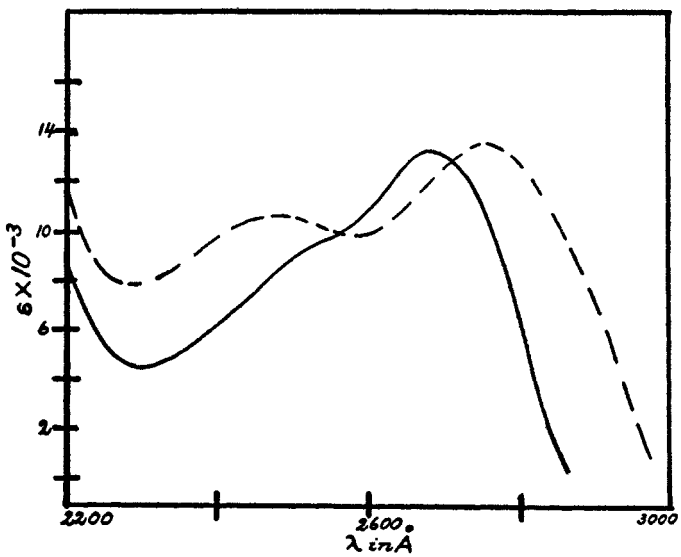


FIGURE 2. ABSORPTION SPECTRUM OF SPONGOSINE. ———— in water and 0.1 *N* NaOH; — — — — in 0.1 *N* HCl.

to the free bases during air-drying, and that in the Florida sponges this process was arrested by the treatment with formalin. More recent observations indicate that the yields of nucleosides obtained by acetone extraction diminish almost to the vanishing point when the sponges are steeped in formalin immediately after their removal from oceanic waters. It appears therefore that the nucleosides are intermediates in the autolysis of more complex products, such as nucleotides or nucleic acids.

It has been pointed out in previous communications of this series (8) that certain lipid components such as fatty acids, sterols, and other unsaponifiable matter occur in lower invertebrates in a diversity far greater than that encountered among animals of higher organization. The results described in the present communication show promise that an analogous variegation will be found among other fundamental constituents of the protoplasm of invertebrates.

If the sponge *Cryptotethia crypta* is not an isolated, unique case, the sponges should prove to be an abundant source of new nucleosides the knowledge of which would be of importance to the understanding of biochemical evolution.

Although the results discussed above offer no convincing proof that the two new nucleosides have ever been parts of larger units such as nucleic acids, they nevertheless point to the probability that the composition of all animal nucleotides and nucleic acids is not necessarily based on the moieties known at present. They also lend weight to the warning voiced by Gulland (9), Chargaff (10), and Davidson (11) against the tacit assumption that all polynucleotides are composed of either the four D-ribose or the four 2-desoxy-D-ribose nucleotides.

The sterols of *Cryptotethia crypta*. The acetone-benzene soluble lipids corresponded to 2–2.5% of the organic matter of the sponge. Upon saponification this fraction gave 56% of unsaponifiable material of which 60% was sterol. By means of fractionation procedures which have been described in preceding communications (12) the mixture was shown to consist of approximately 85% clionasterol and some diunsaturated sterol probably identical with poriferasterol.

EXPERIMENTAL

All melting points are corrected.

Cryptotethia crypta (Florida)

Isolation of spongothymidine. The sponges had first been preserved in formalin and then dried in a vacuum-oven at 60°. Their total ash-content was 40%. Ground sponge material (600 g.), corresponding to about 360 g. of organic material, was extracted with acetone in a modified Soxhlet apparatus (3). During the extraction a nicely crystalline material separated from the boiling solvent. After two days the material was collected (9.01 g.) and triturated with boiling ethanol (400 ml.). The insoluble material (1.5 g.) was dissolved in hot water (20 ml.) and the solution treated with Norit, filtered, and cooled slowly. Clusters of clear prisms were thus obtained, m.p. 246°; $[\alpha]_D^{25°}$ +81.0° (17.9 mg., 3.09 ml. of 8% NaOH, 1 dm., α +0.47°.

Cooling of the alcoholic extract of the first crystalline material afforded another crop of crystals (2.4 g.), m.p. 244°, and further crops were obtained by slow evaporation of the mother liquor. Fractions with similar properties were combined and recrystallized three times from 50% ethanol until constant physical properties were attained. The spongothymidine thus obtained was recrystallized from water, m.p. 246–247°; $[\alpha]_D$ +80.0° (33.5 mg., 3.09 ml. of 8% NaOH, 1 dm., α +0.87°); $[\alpha]_D$ +92.0° (27.3 mg., 3.09 ml. of pyridine, 1 dm., α +0.81°).

Anal. Calc'd for $C_{10}H_{14}N_2O_6$: C, 46.50; H, 5.48; N, 10.85.
Found: C, 46.84; H, 5.42; N, 11.09.

A 59.4-mg. sample of spongothymidine titrated according to the directions of Lythgoe and Todd (6) consumed 49.8 mg. of sodium periodate or a 1.0-molar proportion. A 60.1-mg. sample of adenosine in a parallel titration consumed 48.1 mg. or a 1.0-molar proportion.

Spongothymidine tribenzoate. The nucleoside (150 mg.) was dissolved in 4% sodium hydroxide (2 ml.) and to the solution was added dropwise and with vigorous shaking an excess of benzoyl chloride. The benzoate was separated by centrifugation, washed with water, and recrystallized from methanol, m.p. 190–191°.

Anal. Calc'd for $C_{31}H_{26}N_2O_9$: C, 65.26; H, 4.59; N, 4.91.
Found: C, 65.05; H, 4.75; N, 4.75.

Spongothymidine tri-p-bromobenzoate. To a solution of the nucleoside (140 mg.) in 3.5% sodium hydroxide (2 ml.) was added dropwise and with vigorous shaking a solution of *p*-bromobenzoyl chloride (25 mg.) in benzene (4 ml.). A semisolid precipitate formed which

turned granular overnight. It was washed with water; wt., 222 mg. Repeated recrystallization from ethyl acetate gave rod-shaped crystals melting sharply at 251–252°.

Anal. Calc'd for $C_{31}H_{23}Br_3O_9$: Br, 29.70. Found: Br, 28.74.

Thymine. A solution of the nucleoside (630 mg.) in 10% sulfuric acid was heated in a sealed tube at 130–140° for three hours. It was filtered while hot, to remove black decomposition products, and then cooled, when a copious crop of crystalline material appeared. It was collected, dissolved in methanol, treated with Norit, and recrystallized four times from methanol. When placed in a copper block, preheated to 300°, the thymine melted at 321° with sublimation. It gave no depression of the melting point when mixed with authentic material.

Anal. Calc'd for $C_5H_6N_2O_2$: C, 47.62; H, 4.79; N, 22.21.
Found: C, 47.29; H, 4.15; N, 22.09.

Fractionation of the residues from the mother liquors afforded a small amount of unhydrolyzed nucleoside, m.p. 240°.

Isolation of spongosine. Concentration of the original acetone extract at first gave another crop of spongothymidine and later, upon slow evaporation at room temperature, there separated a material of different appearance. After several recrystallizations from water and ethanol it gave a substance (0.3 g.) of constant m.p. 192–193°; $[\alpha]_D^{25°}$ −42.5° (24.6 mg., 3.09 ml. of 8% NaOH, 1 dm., α −0.34°).

Anal. Calc'd for $C_{11}H_{15}N_3O_5$: C, 44.4; H, 5.08; N, 23.56.
Found: C, 44.36; H, 5.51; N, 23.35.

Hydrolysis of spongosine. A sample of spongosine (120 mg.) was refluxed for one hour with 0.1 *N* sulfuric acid (5 ml.). The material which separated upon cooling was recrystallized several times from dilute ethanol and water, m.p. 278°. The compound was a hydrate. In the sample used for analysis the water had been removed by co-distillation with xylene.

Anal. Calc'd for $C_6H_7N_5O$: C, 43.6; H, 4.3; N, 42.4.
Found: C, 43.4; H, 4.3; N, 42.4.

Deamination of the purine. A sample of the purine was dissolved in warm 0.1 *N* sulfuric acid and the solution treated with sodium nitrite. A reaction took place rapidly and a powdery material separated. It was recrystallized several times from water; needles, m.p. 322–325° on slow heating. The product was dehydrated by treatment with boiling xylene.

Anal. Calc'd for $C_6H_6N_4O_2$: N, 33.8. Found: N, 33.94.

Cryptotethia crypta (Bahamas)

The sponges were air-dried, ground, and extracted with acetone. From 620 g. of material, corresponding to 280 g. of organic matter, was obtained about 4 g. of a semi-crystalline material which had separated from the boiling acetone. It was washed thoroughly with benzene and then extracted from a thimble with acetone. The material obtained from the acetone extract melted indefinitely above 270°. It was slightly levorotatory and its nitrogen content was in excess of 20%. These data indicated that the mixture contained little if any spongothymidine.

When a solution of the mixture in 0.1 *N* sulfuric acid was refluxed for several hours and then cooled, a copious yield of a crystalline material was obtained which was shown to be thymine, m.p. 320–321° (slow heating). Silver acetate was added to the mother liquor and the precipitated silver salt was washed with water and decomposed with hydrogen sulfide. A product was thus obtained which proved to be identical with the purine formed upon hydrolysis of spongosine.

Isolation of the sterols of Cryptotethia crypta. The acetone mother liquors from the extractions of the Florida or Bahamas sponges were evaporated to dryness and the residue was dissolved in benzene. The solution was filtered and evaporated to dryness. Lipid fractions were thus obtained in yields corresponding to 2–3% of the organic matter of the sponges. Upon saponification they gave an average yield of 56% of unsaponifiable material which, on the average, contained 60% of sterols.

The crude sterols were at once acetylated. Upon titration with perbenzoic acid various acetate fractions showed the presence of 1.05–1.2 double bonds. The acetates were dissolved in ether and treated with a 5% solution of bromine in acetic acid. The bromides thus obtained were separated by trituration with ether into an insoluble tetrabromide and a soluble dibromide fraction as previously described (12). Only a small amount of tetrabromide, m.p. 187°, was obtained which appeared to be identical with proiferasteryl acetate tetrabromide.

The soluble bromides were debrominated and the acetate recrystallized several times from a mixture of chloroform-methanol and from ethanol, m.p. 134–135°; $[\alpha]_D^{25}$ −42.5° (30.8 mg., 3.06 ml. of chloroform, 1 dm., α −0.43°). It gave no depression of the melting point when mixed with authentic clionasteryl acetate.

Saponification of the acetate gave clionasterol, m.p. 137.5–138.5°, $[\alpha]_D^{25}$ −37.0° (33.1 mg., 3.06 ml. of chloroform, 1 dm., α −0.40°). The sterol was converted to clionasteryl propionate, m.p. 116–116.5°, $[\alpha]_D^{25}$ −41.3° (30.7 mg., 3.06 ml. of chloroform, 1 dm., α −0.41°) and clionasteryl benzoate, m.p. 139–139.5°, $[\alpha]_D^{25}$ −17.0° (31.4 mg., 3.06 ml. of chloroform, 1 dm., α −0.175°). None of the compounds gave depression of their melting points when mixed with authentic material.

SUMMARY

1. Two new nucleosides have been isolated from the sponge *Cryptotethia crypta* which had been collected near the coast of Florida.

2. One of the nucleosides has been shown to be a pentofuranosylthymine by its elementary analysis, its hydrolysis to thymine, its formation of a tribenzoate and tri-*p*-bromobenzoate, and by titration with sodium periodate. The trivial name spongothymidine has been proposed for this nucleoside.

3. Another nucleoside, for which the trivial name spongosine has been proposed, has been shown to be a pentosylmethylaminoöxypurine. The purine has been isolated and converted to a methyldioxypurine.

4. The isolation of thymine and a methylaminoöxypurine from the acetone extract of *Cryptotethia crypta*, collected in the coastal waters of the Bahamas, has been described.

5. It has been shown that the sterol mixture from *Cryptotethia crypta* consists principally of clionasterol.

NEW HAVEN, CONNECTICUT

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