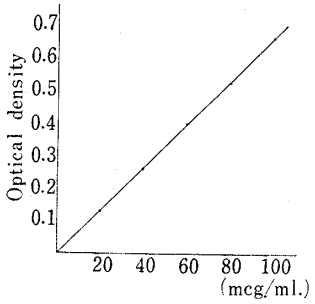

Fig. 5. Standard Curve for Spectrophotometric Determination of Cycloheximide

One ml. of various concentration of Cycloheximide solution, 2 ml. of 10% resorcinol and 2 ml. of conc.HCl were mixed, heated for 30 min. in boiling water bath, cooled, readjusted to 5 ml., and measured at 400 mμ vs. a reagent blank similarly prepared.

The percent cycloheximide in the sample is obtained by calculating from the standard curve (Fig. 5) similarly prepared using 1 ml. of 20 to 100 mcg./ml. cycloheximide standard solution, standard curve being expressed by the following equation.

$$\text{Optical density} = -0.004 + 0.00665 \times \text{concentration (mcg./ml.)}$$

(Standard error of estimate : 0.004)

It must be added that this colorimetric procedure is interferred in the cases when the cycloheximide preparation contains the contaminants like carbohydrates which are sensitive to this color reagent.

The authors express their gratitude to Dr. K. Abe, the Superintendant of this Laboratory, for his grant of publication. The authors' thanks are also indebted to Dr. A. Kiyomoto, Dr. M. Suzuki and Mr. Y. Egawa, for their instructive suggestions.

Summary

A new and simple procedure is described for the spectrophotometric determination of cycloheximide and its related compounds, using resorcinol and hydrochloric acid as colorimetric reagents.

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48. Mitsuji Sano : Studies on Nucleosides and Nucleotides. III.*1 Synthesis of Glycosyl-2-thiouracils from Glycosylthioureas.

(*Research Laboratory, Daiichi Seiyaku Co., Ltd.**2)

A new method for synthesis of glycosyl-2-thiothymine from glycosylthiourea was established and reported in Part II of this series.*1 Later, this method was extended to the synthesis of glycosyl-2-thiouracil, and 1-(β-D-glucopyranosyl)-2-thiouracil*3 and 1-(β-D-ribofuranosyl)-2-thiouracil (2-thiouridine) were successfully synthesized.

Before carrying out the present synthesis, preliminary experiment was carried out for preparation of 1-methyl-2-thiouracil from N-methylthiourea and, 3-ethoxyacryloyl

*1 Part II : This Bulletin, **9**, 709 (1961).
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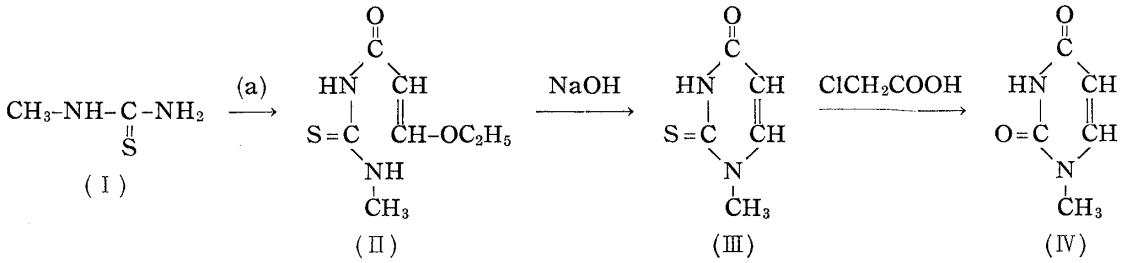
*3 Nomenclature of uracils used in the present paper followed that used in the Chemical Abstracts.

chloride, similarly as in the preparation of 1-methyl-2-thiothymine from 1-methylthiourea and 2-methyl-3-methoxyacryloyl chloride described in Part I of this series.[1] Synthesis of glycosyl thiouracil was then examined.

Condensation of 1-methylthiourea (I) and 3-ethoxyacryloyl chloride (a) in chloroform, with pyridine as the deacidification agent, afforded a product (II) of m.p. 122~124°, identical with 1-(3-ethoxyacryloyl)-3-methylthiourea, prepared by Shaw and Warrener[2] from 3-ethoxyacryloyl isothiocyanate. Cyclization of (II) with sodium hydroxide or ammonia gave 1-methyl-2-thiouracil (III), m.p. 226~228°, which was identified by mixed fusion with 1-methyl-2-thiouracil synthesized by a different route according to the method of Shaw.[2] Treatment of (III) with monochloroacetic acid converted it to 1-methyluracil, m.p. 230~232°.

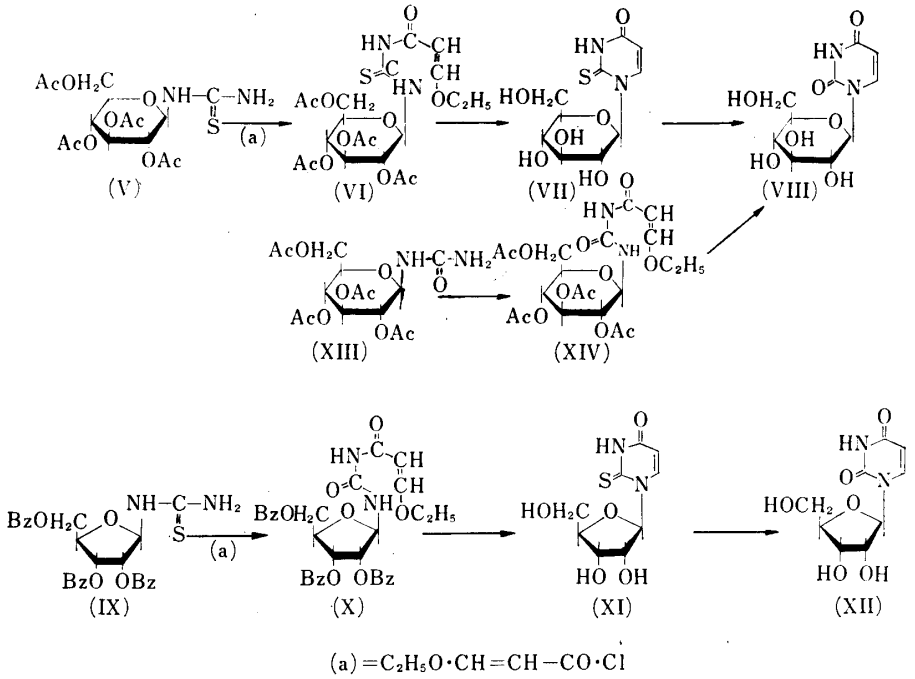


The foregoing preliminary experiment suggests the possibility of obtaining glycosyl-2-thiouracil by application of 3-ethoxyacryloyl chloride to glycosylthiourea and the synthesis of 1-(β-D-glucopyranosyl)-2-thiouracil was first taken up.

Tetra-O-acetyl-β-D-glucopyranosylthiourea*[1] (V) and 3-ethoxyacryloyl chloride in chloroform, with pyridine as the deacidification agent, were allowed to stand over night at room temperature and refluxed for 2 hours. The product was purified through silica gel chromatography and 1-(tetra-O-acetyl-3-D-glucopyranosyl)-3-(3-ethoxyacryloyl)-thiourea (VI) was obtained as the intermediate. Since this substance did not crystallize, it was submitted to cyclization, per se, by warming with dilute ammonia water. The product was purified by chromatography using activated charcoal, according to the method of Stambaugh,[3] but the product could not entirely be separated from the starting glucosylthiourea. The product was therefore submitted to partition chromatography through a column of Celite 535, with methyl ethyl ketone as the eluant, and separation was effected, affording 1-(β-D-glucopyranosyl)-2-thiouracil (VII), m.p. 226~227°. This substance was reported by Fox, *et al.*[4] as having been prepared from tetra-O-acetyl-D-glucosyl halide and silver salt of 2-thiouracil but they stated that the yield was low and failed to give any detail on the melting point and other points.

Treatment of (VII) with monochloroacetic acid gave 1-β-D-glucopyranosyluracil (VIII) of m.p. 207~209°, which agreed well with the melting point noted for the substance synthesized by Hilbert and others.[5] This substance (VIII) was also identified by mixed fusion with 1-β-D-glucopyranosyluracil obtained by cyclization of 1-(tetra-O-acetyl-β-D-glucopyranosyl)-3-(3-ethoxyacryloyl)urea (XIV), formed by reaction of tetra-O-acetyl-β-D-glucopyranosylurea (XIII) and 3-ethoxyacryloyl chloride (a) in chloroform, with pyridine as the deacidification agent, same as in the case of thiourea, and subsequent purification by alumina chromatography.

1) Part I: This Bulletin, **9**, 703 (1961).
2) G. Shaw, R. Warrener: J. Chem. Soc., **1958**, 153.
3) R.L. Stambaugh: J. Chromatog., **3**, 22 (1960).
4) J.J. Fox, N. Chang, J. Davoll: Federation Proc., **13**, 211 (1954).
5) G. Hilbert, T.B. Johnson: J. Am. Chem. Soc., **52**, 4489 (1930).



(a) $=C_2H_5O\cdot CH=CH-CO\cdot Cl$

Synthesis of 1-(β-D-ribofuranosyl)-2-thiouracil was then carried out. This substance had already been obtained by Strominger and others[6] from ribose phosphate and thiouracil by biosynthesis with thymidine phosphorylase and by Shaw and others[7] from tri-O-benzoyl-D-ribofuranosylamine. In the present work, 1-(tri-O-benzoyl-3-D-ribofuranosyl)thiourea*[1] (IX), synthesized in an earlier work, was allowed to stand with 3-ethoxyacryloyl chloride in chloroform, with pyridine as the deacidification agent, for 3 days at room temperature, refluxed for 2 hours, and the product was purified by silica gel chromatography, affording amorphous 1-(tri-O-benzoyl-β-D-ribofuranosyl)-3-(3-ethoxyacryloyl)-thiourea (X). This was heated with methanolic ammonia in a sealed tube at 100° and the product was purified by chromatography through activated charcoal according to the method of Stambaugh,[8] from which 1-(β-D-ribofuranosyl)-2-thiouracil (2-thiouridine) (XI) was obtained. This substance was identified by mixed fusion with the compound synthesized by the method of Shaw.[7]

Attempted conversion of (XI) to uridine (XII) by treatment with monochloroacetic acid and paper chromatography of the product showed that a large amount of unreacted material (XI) remained, although there was evidence that the objective substance was formed in a small amount, by comparison with the chromatogram of natural uridine.

The foregoing experiments have shown that the biochemically interesting 1-glycosyl-2-thiouracils can be synthesized by the same method as for preparation of 1-glycosyl-2-thiothymine.

Experimental

1-Methyl-3-(3-ethoxyacryloyl)thiourea (II)——To 0.9 g. (0.01 mole) of 1-methylthiourea (I) suspended in 15 cc. of $CHCl_3$, 1.0 g. (0.012 mole) of pyridine and 1.6 g. (0.012 mole) of 3-ethoxyacryloyl chloride were added and the mixture was refluxed on a water bath for 2.5 hr. The pale yellow, translucent reaction mixture was cooled, washed with aqueous solutions of $NaHSO_4$ and $NaHCO_3$, and water, dried over anhyd. Na_2SO_4, and $CHCl_3$ was evaporated in reduced pressure. EtOH was added to the residue, evaporated, and a small amount of EtOH was again added to the residue. The crystals

6) D. Strominger, M. Friedkin: J. Biol. Chem., **208**, 663 (1954).
7) G. Shaw, R. Warrener, M. Maguire: J. Chem. Soc., **1958**, 2294.

that separated out were collected and recrystallized from EtOH to 0.6 g. of colorless scales, m.p. 122~124°. *Anal.* Calcd. for $C_7H_{12}O_2N_2S$: C, 44.68; H, 6.43; N, 14.88. Found: C, 44.56; H, 6.58; N, 14.65.

1-Methyl-2-thiouracil (III)——Prepared according to the method of Shaw.[2] A mixture of 0.4 g. of (II) and 4 cc. of 2*N* NaOH was warmed on a water bath for 10 min. to effect solution, cooled, and acidified with HCl. The crystals that precipitated out were collected, washed with water, and dried to 0.25 g. of crystals. Recrystallization from EtOH afforded 0.2 g. (66%) of colorless needles, m.p. 226~228°, same as that reported by Shaw.

1-Methyluracil (IV)——A mixture of 0.3 g. of (III), 2 g. of monochloroacetic acid, and 20 cc. of water was refluxed in an oil bath for 10 hr. and the reaction mixture was evaporated to dryness in reduced pressure. A small amount of EtOH was added to the syrupy residue and the mixture was allowed to stand, by which crystals were separated out. Recrystallization from EtOH afforded 0.2 g. (75%) of colorless needles, m.p. 230~232°, agreeing with the m.p. recorded by Shaw and others.[8]

1-(β-D-Glucopyranosyl)-2-thiouracil (VII)——To a suspension of 2.4 g. (0.006 mole) of 1-(tetra-O-acetyl-β-D-glucopyranosyl)thiourea (V) suspended in 15 cc. of dehyd. $CHCl_3$, 0.65 g. (0.007 mole) of pyridine and 0.8 g. (0.006 mole) of 3-ethoxyacryloyl chloride were added. There was slight generation of heat and the mixture was cooled with water. After allowing the mixture to stand in an ice chamber over night, it was refluxed on a water bath for 2 hr., cooled, and washed with aqueous solutions of $NaHSO_4$ and $NaHCO_3$, and water. The solution was dried over anhyd. Na_2SO_4, $CHCl_3$ was evaporated in reduced pressure, and 3.0 g. of pale yellow syrup was obtained.

This syrup was dissolved in 20 cc. of $CHCl_3$ and the solution was passed through a column (diam., 3.5 cm.) of 100 g. of silica gel (100~200 mesh) and $CHCl_3$. The column was eluted consecutively with 400 cc. of $CHCl_3$, 400 cc. of $CHCl_3$-AcOEt (9:1), 200 cc. of $CHCl_3$-AcOEt (4:1), and 2000 cc. of $CHCl_3$-AcOEt (1:1), and the effluent was fractionated into 90 fractions of each 25 cc. The presence of eluted substance was examined*3 in each fraction and the combined fraction Nos. 37~47 containing the main eluate was evaporated to dryness, affording 1.7 g. (57%) of pale yellow, amorphous 1-(2′,3′,4′,6′-tetra-O-acetyl-β-D-glucopyranosyl)-3-(3-ethoxyacryloyl)thiourea (VI). UV: $\lambda_{max}^{H_2O}$ 273.5 mμ.

A mixture of 1.7 g. of (VI) and 90 cc. of 3.3% NH_4OH solution was stirred with warming on a water bath for 1.5 hr. to effect solution, the reaction mixture was treated with a small amount of activated charcoal, and the liquid was evaporated to dryness to leave 1.3 g. of syrup. The paper chromatography of this residue, developed with MeCOEt, showed a spot detected by the ultraviolet ray (2536 Å) at Rf 0.20, considered to be that of (VII) and also that of the starting glucosylthiourea at Rf 0.06.

This syrup was purified by chromatography with activated charcoal, using the apparatus shown in Fig. 2 of Part II,*1 with a column of 3.5 cm. in diameter and a mixture of 30 g. of activated charcoal and 30 g. of cellulose powder. A solution of 1.3 g. of the syrup dissolved in 20 cc. of distilled water was adjusted to pH 2 with dil. HCl and passed through this column. The column was washed with distilled water until the effluent no longer showed acid reaction. In the upper part (A in Fig. 2 of Part II) and B, 200 and 800 cc. of distilled water were placed and the column was developed by a solvent in C, containing 5:1:13 mixture of conc. NH_4OH, EtOH, and H_2O. The effluent was fractionated into 50 fractions of each 25 cc. and the presence of the product in the eluate was examined in each fraction.*3 The fraction containing an eluted substance was examined by paper partition chromatography and the fractions showing a single spot at Rf 0.20 were collected.

Such a fraction was, however, found to be contaminated with the starting material and the combined fraction was submitted to partition chromatography, using Celite 535 and MeCOEt. A mixture of 7 g. of Celite 535 and 7 cc. of distilled water saturated with MeCOEt was allowed to stand, suspended in MeCOEt, saturated with water and filled in a chromatographic tube of 2 cm. in diameter. The sample mixed with Celite was placed on top of the column and partitioned with water-saturated MeCOEt into 35 fractions of each 20 cc. Complete separation was thereby effected, the effluent containing (VII), showing a spot at Rf 0.20, being found in fraction Nos. 7~13 and that containing glucosylthiourea in fraction Nos. 20~27. The combined fraction Nos. 7~13 was evaporated to dryness in reduced pressure and addition of MeOH to the residue afforded 160 mg. (16%) of crystals. Recrystallization from MeOH gave colorless prisms, m.p. 226~227°. $[\alpha]_D^{20}$ +18.5° (c=1.46, H_2O). UV: $\lambda_{max}^{H_2O(pH\ 5.6}$ 278 mμ (log ε 4.22). *Anal.* Calcd. for $C_{10}H_{14}O_6N_2S \cdot 2CH_3OH$: C, 40.67; H, 6.26; N, 7.91. Found: C, 40.86; H, 5.78; N, 8.17.

Formation of 1-β-D-Glucopyranosyluracil (VIII) from 1-(β-D-Glucopyranosyl)-2-thiouracil——A mixture of 50 mg. of (VII), 1 g. of monochloroacetic acid, and 10 cc. of water was refluxed for 6 hr. The reaction mixture was purified by chromatography with activated charcoal, used for the purification of (VII). In a chromatographic tube of 2 cm. in diameter, 1.2 g. each of activated charcoal

*3 The effluent was spotted on a filter paper, dried, and the paper was irradiated with ultraviolet ray (2536 Å), by which the product would be detected as a dark purple spot.

8) G. Shaw, R. Warrener: J. Chem. Soc., **1958**, 157.

charcoal and cellulose powder were placed, 50 cc. and 100 cc. of water in the upper part of A and B, and effluent was collected in 5-cc. fractions. Fraction Nos. 11～15 contained a substance which showed a spot at Rf 0.11 (water-saturated BuOH). The combined fraction Nos. 11～15 was evaporated to dryness in reduced pressure and addition of EtOH to the residue resulted in crystallization. Recrystallization from MeOH gave 25 mg. (53%) of colorless prisms, m.p. 197～202° (decomp.). The sample, dried at 115～140° in reduced pressure (1 mm. Hg), melted at 207～209°. $[\alpha]_D^{20}$ +20.9° (c=1.35, H_2O). UV : $\lambda_{max}^{H_2O(pH\ 6.5)}$ 258 mμ (log ε 3.98). *Anal.* Calcd. for $C_{10}H_{14}O_7N_2$: C, 43.80; H, 5.15; N, 10.22. Found: C, 43.36; H, 5.54; N, 9.75.

1-(2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranosyl)-3-(3-ethoxyacryloyl)urea (XIV)——To a solution of 1.8 g. (0.015 mole) of tetra-O-acetyl-β-D-glucopyranosylurea in 2.5 cc. of dehyd. $CHCl_3$, 0.6 g. (0.017 mole) of 3-ethoxyacryloyl chloride and 0.35 cc. of pyridine were added and the mixture was allowed to stand for 24 hr. at room temperature. The reaction mixture was evaporated to dryness in reduced pressure and the residue was recrystallized from AcOEt, but the product did not crystallize.

The residue was purified by column chromatography over 20 g. of alumina and the column was developed with AcOEt and AcOEt-$CHCl_3$ mixture, collecting fractions absorbing ultraviolet ray. The effluent was concentrated, petr. ether was added to the residue, and the crystals that formed were recrystallized from MeOH to 0.1 g. of colorless needles, m.p. 151°. The mother liquor afforded further crop of 0.31 g. of crude crystals. Combined yield, 13.7%. $[\alpha]_D^{22}$ −40.94° (c=0.701, $CHCl_3$). UV : λ_{max} 253 mμ (log ε 4.347). *Anal.* Calcd. for $C_{20}H_{28}O_{12}N_2$: C, 48.97; H, 6.16; N, 5.72. Found: C, 49.46; H, 5.86; N, 5.63.

1-(β-D-Glucopyranosyl)uracil (VIII)——A solution of 0.31 g. of (XIV) dissolved in a small quantity of EtOH, added with 9 cc. of 3.3% NH_4OH, was heated at 80° for 2.5 hr., the reaction mixture was evaporated to dryness, and EtOH was added to the residue. EtOH solution was evaporated, the residue was dissolved in a small quantity of EtOH, and AcOEt was added to it until persistent white turbidity appeared. Crystals appeared on standing this mixture and the colorless prisms so obtained, dried at 140～150° in reduced pressure, melted at 198～200°, showing no depression on admixture with 1-β-D-glucopyranosyluracil obtained from (VII). *Anal.* Calcd. for $C_{10}H_{14}O_7N_2 \cdot \frac{1}{2}H_2O$: C, 42.40; H, 5.34; N, 9.89. Found : C, 42.94; H, 5.12; N, 9.87.

2-Thiouridine (XI)——To a suspension of 2.0 g. (0.0038 mole) of 1-(tri-O-benzoyl-β-D-ribofuranosyl)-thiourea*1 (IX) in 10 cc. of $CHCl_3$ dried over P_2O_5, 0.45 cc. (0.0057 mole) of pyridine and 0.7 g. (0.0038 mole) of β-ethoxyacryloyl chloride were added, by which the thiourea compound dissolved readily with slight evolution of heat. The mixture was allowed to stand at room temperature for 3 days and refluxed for 2 hr. The mixture was washed consecutively with aqueous solutions of $NaHSO_4$ and $NaHCO_3$, and water, dried over anhyd. Na_2SO_4, and $CHCl_3$ was evaporated in reduced pressure to leave 4.2 g. of a syrup.

This syrupy residue was dissolved in 20 cc. of $CHCl_3$ and passed through a column (3.5×30 cm.) of 130 g. of silica gel (100～200 mesh) suspended in $CHCl_3$. The column was developed with 600 cc. of $CHCl_3$ and 4000 cc. of $CHCl_3$-AcOEt (9:1) mixture, and the effluent was collected in 20-cc. fractions until the effluent no longer contained any eluted substance. The main eluate was found in the fraction Nos. 29～56 which were combined and evaporated in reduced pressure to leave 1.9 g. of a syrup. Trituration of the syrup with petr. ether afforded an amorphous substance assumed to be 1-(tri-O-benzoyl-β-D-ribofuranosyl)-3-(3-ethoxyacryloyl)thiourea (X).

A mixture of 1.6 g. of this amorphous product and 30 cc. of 3% MeOH-NH_3 was heated in a sealed tube at 100° for 2 hr. and the reaction mixture was evaporated in reduced pressure to leave a syrup. Paper partition chromatography of this syrupy residue gave a spot*3 of (XI) at Rf 0.36 (BuOH saturated with 3% H_3BO_3 solution) or at Rf 0.51 (BuOH-AcOH-H_2O=5:2:3) and its separation was effected by chromatography through activated charcoal, using the apparatus indicated in Fig. 2 in Part II.*1 The chromatographic tube of 3.5 cm. in diameter was filled with 1.2 g. of each activated charcoal and cellulose powder, 50 cc. and 300 cc. of distilled water were placed respectively in upper part of A and in B, and 800 cc. of 5:1:13 mixture and 1000 cc. of 5:5:13 mixture of conc. NH_4OH-EtOH-H_2O were placed in C to effect consecutive development. Effluent was collected in 20-cc. fractions and paper partition chromatography of each fraction showed the presencc of the main product in the fraction Nos. 44～56. These fractions were combined, evaporated in reduced pressure, and crystals that separated out were recrystallized from hydrous EtOH to 150 mg. (15%) of colorless prisms, m.p. 212～214°. $[\alpha]_D^{20}$ +38.5° (c=1.4, H_2O). UV : $\lambda_{max}^{H_2O(pH\ 6.5)}$ 274 mμ (log ε 4.14) *Anal.* Calcd. for $C_9H_{12}O_5N_2S$: C, 41.55; H, 4.65; N, 10.75. Found : C, 41.35; H, 4.57; N, 10.78.

Formation of Uridine (XII) from 2-Thiouridine (XI)——A mixture of 50 mg. of (XI), 1 g. of monochloroacetic acid, and 10 cc. of water was refluxed for 5 hr. Paper partition chromatography of the reaction mixture showed a small spot of uridine at Rf 0.41 (BuOH-AcOH-H_2O=5:2:3) and a large spot of the starting 2-thiouridine at Rf 0.50. When developed with a solvent system of BuOH saturated with 3% H_3BO_3 solution, the spot of uridine appeared at Rf 0.19 and that of 2-thiouridine at

Rf 0.33, the spot of uridine being extremely small. Further extension of the reaction failed to increase the amount of uridine.

The author expresses his gratitude to Prof. T. Ukita of the Faculty of Pharmaceutical Sciences, University of Tokyo, for reviewing this manuscript, and to the members of the Laboratory of Hygiene and Forensic Chemistry of the said University for valuable discussions. He is grateful to Dr. J. Shinoda, the President, Dr. T. Ishiguro, Director of the Laboratory, and Dr. M. Shimizu, Acting Director of the Laboratory, all of this Company, for giving permission to publish this work. He is indebted to Dr. T. Naito for unfailing and kind guidance throughout the course of the present work and to Messrs. B. Kurihara and K. Abe for elemental analytical data.

Summary

Synthesis of glycosylthiouracil from glycosylthiourea was carried out by the method for synthesis of glycosylthymine described in Part II of this series. 1-Methyl-3-(3-ethoxyacryloyl)thiourea, obtained from N-methylthiourea and ethyl 3,3-diethoxyacryloyl chloride, was submitted to pyrimidine cyclization and 1-methyl-2-thiouracil was found to be produced. In accordance with this model experiment, 1-(β-D-glucopyranosyl)-2-thiouracil was obtained from 1-(tetra-O-acetyl-β-D-glucopyranosyl)thiourea and ethyl 3,3-diethoxyacryloyl chloride, and 1-(β-D-ribofuranosyl)-2-thiouracil (2-thiouridine) from 1-(tri-O-benzoyl-β-D-ribofuranosyl)thiourea.

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49. Mitsuji Sano : Studies on Nucleosides and Nucleotides. IV.*1
Synthesis of Isoglycosyluracils and Isoglycosyl-2-thiouracils from Glycosylureas and Glycosylthioureas.

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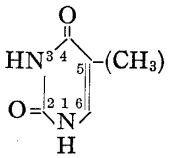
Naito and others reported in Part I of this series[1] on a new method for synthesis of pyrimidine nucleoside and assumed that 3-glycosyl-2-thiothymine (pyrimidines with a glycosyl group in 3-N position will tentatively designated hereafter as isopyrimine nucleoside) would be obtained from N-glycosylthiourea by application of this method, since, in preliminary experiment, condensation of N-methylthiourea and ethyl 2-formylpropionate afforded 3-methyl-2-thiothymine.*3 Based on this assumption, reaction of glycosylthiourea and ethyl 2-formylpropionate or ethyl 3,3-diethoxypropionate was examined and isopyrimidine nucleoside was successfully obtained.

Isopyrimidine nucleosides are unknown substances and are extremely bitter compounds, possessing a glycosyl group bonded to the nitrogen in 3-position of the pyrimidine

*1 Part III : This Bulletin, **9**, 308 (1961).
*2 Minamifunabori-cho, Edogawa-ku, Tokyo (佐野光司).
*3 Nomenclature of uracil and thymines in this paper followed that used in the Chemical Abstracts, i.e.



1) Part I : This Bulletin, **9**, 703 (1961).