Associates A-60 spectrometer, using deuteriochloroform as solvent and tetramethylsilane as internal reference. The melting points were taken on a Fisher-Johns block and are uncorrected. Microanalyses were performed in the laboratory of Dr. A. Bernhardt, Mülheim, Germany. All solvents were distilled before they were used.

Isolation.—Extraction of 75 lb. of dry seeds of *Mammea americana* L. with Skellysolve B provided 1.6 kg. of mamey oil after the removal of all acetone-insoluble material. Twenty grams of this extract was chromatographed on 1375 g. of aluminum oxide (Merck, acid washed). The column was eluted with Skellysolve B using an increasing ratio of benzene. Fractions of 500 ml. were taken.

Mammeol.—The first crystalline compound, mammeol, was obtained from a yellow wax (fractions 135–151), eluted with Skellysolve B–benzene (1:1). Several crystallizations of this material from hexane afforded 15 mg. (3.55×10^{-3}% yield, based on dry seeds) of white crystals: m.p. 148–150°; ν_{max}^{KBr} 3450, 2970, 1455, 1378, 1066, 968, and 979 cm.$^{-1}$.

Anal. Calcd. for $C_{20}H_{34}O$: C, 82.69; H, 11.80; O, 5.51; mol. wt., 290. Found: C, 82.29, 82.87; H, 11.69, 11.89; O, 5.56; mol. wt., 320.

Mammeigin (3).—From fractions 210–219, eluted with Skelly solve B–benzene (1:9), 237 mg. of a partly crystallized yellow oil was obtained. Separation of the crystalline material from the oil on an aluminum oxide column (Woelm, neutral, activity I) proved futile. Up to 30 crystallizations from ether–hexane mixtures afforded 58 mg. (1.37×10^{-2}% yield, based on dry seeds) of yellow needles: m.p. 144–146°; ν_{max}^{KBr} 3440 (broad), 1746, 1644, 1613, 1126, 773, 752, and 708 cm.$^{-1}$; $\lambda_{max}^{95\%\ ethanol}$ 234, 286, and 365 mμ (log ε 4.45, 4.52, and 4.11); $\lambda_{max}^{95\%\ ethanol-NaOH}$ 218, 251, 312, and 438 mμ (log ε 4.33, 4.38, 4.41, and 3.84). The compound gave a green color with ferric chloride.

Anal. Calcd. for $C_{25}H_{24}O_5$: C, 74.24; H, 5.98; O, 19.78; mol. wt., 404. Found: C, 74.34, 74.04; H, 6.15, 6.34; O, 19.81; mol. wt., 401.

The n.m.r. spectrum is reproduced in Figure 3. The relative integral was measured as follows: τ −4.17 (1), 2.94 (5.4), 3.33 (1.1), 4.34 (1), 4.59 (0.8), 7.14 (2.1), 7.72–8.33 (0.8), 8.48 (6.3), and 9.06 (6.2).

Dihydromammeigin (4) from Mammeigin (3).—Mammeigin (50 mg.) was stirred in 3 ml. of ethanol with 5% palladium on carbon (10 mg.) in a hydrogen atmosphere at 25°. The compound was not completely soluble in the amount of solvent which was limited by the size of the microhydrogenator. After 2 hr. 0.4 molar equiv. of hydrogen was taken up and further consumption occurred at a very slow pace. Ethanol was removed by a nitrogen jet and replaced by 3 ml. of tetrahydrofuran (distilled from $LiAlH_4$). Additional catalyst (5 mg.) was introduced and the hydrogen uptake ceased after 20 min. The total uptake was 0.97 molar equiv. Filtration of the reaction mixture and evaporation of the solvent afforded a yellow semisolid. Crystallization from a tetrahydrofuran–hexane mixture yielded 42 mg. of tan crystals, m.p. 152–160°. Three additional recrystallizations raised the melting point to 164–165° (25 mg.). From mother liquors, 11 mg., m.p. 154–161°, was obtained. A mixture melting point with a sample of dihydromammeigin, m.p. 165–166°, obtained on cyclization of mammeisin showed no depression, m.p. 164–166°. The infrared spectra of the two samples were superimposable, and their n.m.r. spectra showed identical shifts and coupling constants.

Dihydromammeigin (4) from Mammeisin (2).—A sample of 250 mg. of mammeisin was dissolved in 5 ml. of glacial acetic acid and 3 drops of concentrated sulfuric acid was added. After standing for 18 hr. at room temperature, the reaction mixture was poured onto ice–water. A tan amorphous material precipitated. The filtered solid was dissolved in ether, washed several times with water, and dried over magnesium sulfate. Removal of the ether afforded 233 mg. of tan crystals, m.p. 146–158°. Three further recrystallizations from tetrahydrofuran–ether–hexane mixtures yielded tan needles: m.p. 165–166°; ν_{max}^{KBr} 3436 (broad), 1745, 1730 (sh), 1613, 1587 (sh), 1124, 770, and 714 cm.$^{-1}$; $\lambda_{max}^{95\%\ ethanol}$ 287 and 341 mμ (log ε 4.54 and 4.05); $\lambda_{max}^{95\%\ ethanol-NaOH}$ 293, 321, and 420 mμ (log ε 4.07, 4.12, and 3.89).

Anal. Calcd. for $C_{25}H_{26}O_5$: C, 73.87; H, 6.45; O, 19.68. Found: C, 73.78; H, 6.60; O, 19.56.

Acknowledgment.—The technical assistance of Mrs. U. Mueller, Mr. G. Hogsten, and Mr. B. A. Modi is gratefully acknowledged.

Analogs of Firefly Luciferin

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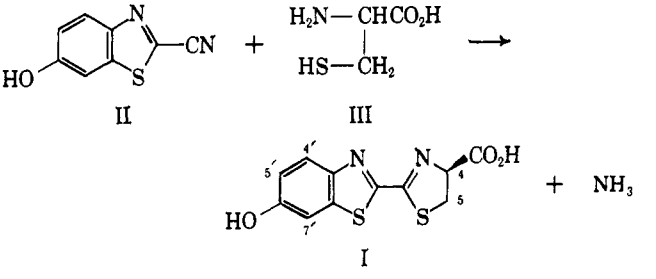
The synthesis of firefly luciferin (and the C^{14}- and S^{35}-labeled forms) is outlined. In addition, the syntheses of O-methylluciferin, 5,5-dimethylluciferin, deshydroxyluciferin, decarboxyluciferin, and a pyridine analog are reported.

Recently we showed by degradation and synthesis that luciferin, the oxidizable substrate responsible for light emission in the firefly, is D-(−)2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (I).[1] We have since synthesized a number of analogs in an effort to determine the structural requirements for firefly luciferin in bioluminescence; these syntheses are the subject of this paper.

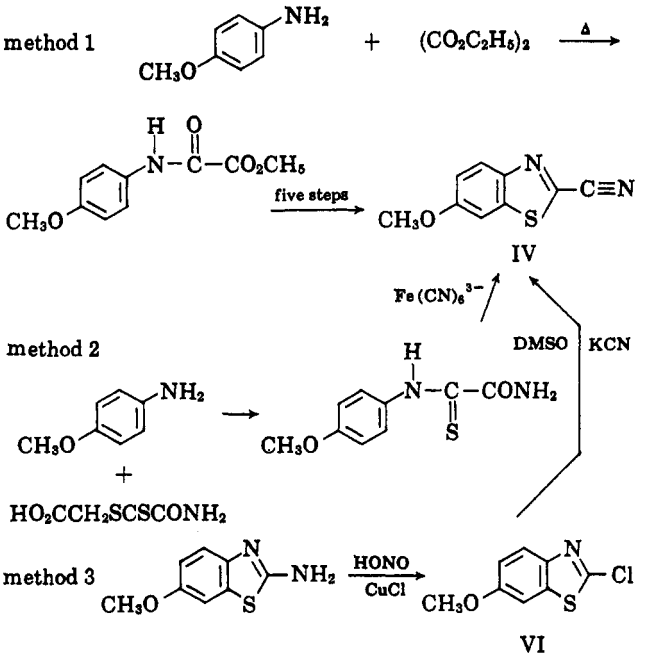
Firefly Luciferin (I).—The final and key step in our earlier synthesis of luciferin[1] was the condensation of 2-cyano-6-hydroxybenzothiazole (II) with cysteine (III); aqueous methanol was used as the solvent, and the condensation was effected at room temperature.[2] Compound II was prepared, in turn, by



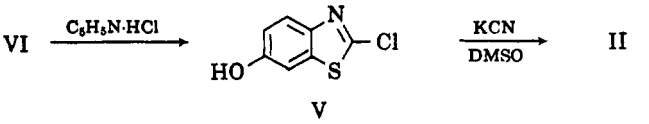
the demethylation of 2-cyano-6-methoxybenzothiazole (IV). This compound has now been prepared by three different routes. Method 1, requiring six steps, has been largely replaced by method 3 in which a commercially available intermediate containing the benzothiazole nucleus is used. Method 3 also lends itself to the synthesis of C^{14}-containing luciferin (see Experimental) since potassium cyanide-C^{14} is readily available. Method 2, introduced recently by Japanese

(1) E. H. White, F. McCapra, and G. F. Field, *J. Am. Chem. Soc.*, **85**, 337 (1963); E. H. White, F. McCapra, G. F. Field, and W. D. McElroy, *ibid.*, **83**, 2402 (1961).

(2) A similar condensation has also been reported by H. Baganz and L. Domaschke, *Ber.*, **95**, 1842 (1962).

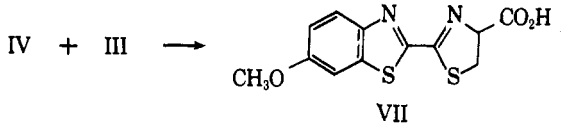


workers,[3] is convenient for the synthesis of large amounts of luciferin. In an additional variation of the synthesis, compound VI was demethylated, and the product was condensed with potassium cyanide-C^{14}



to give 2-cyano-6-hydroxybenzothiazole (II) directly. Modified directions for the synthesis of luciferin and the n.m.r. data for this compound in deuterium oxide are given in the Experimental section.

O-Methylluciferin (VII).—2-Cyano-6-methoxybenzothiazole (IV), which in the synthesis of firefly luciferin is converted into the hydroxynitrile II, was now condensed directly with cysteine. The product, 2-(6′-methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic



acid (VII), a monobasic analog of firefly luciferin, was obtained in high yield. The n.m.r. spectrum in deuterioacetone was similar to that of luciferin[1]: the 4′-proton appeared as a doublet at τ 2.00 ($J_{4',5'}$ = 9 c.p.s.), the 7′-proton as a doublet at τ 2.37 ($J_{5',7'}$ = 2.5 c.p.s.), the 5′-proton as a pair of doublets (centered at τ 2.80), the 4-proton as a triplet centered at τ 4.53 (with the symmetrical appearance of the X part of an A_2X spectrum, J = 9 c.p.s.), the methyl protons as a singlet at τ 6.08, and the 5-hydrogens as a multiplet centered at about τ 6.2 and partially obscured by the methyl signal; the three hydrogens at positions 4 and 5 presumably constitute an ABX system.[4]

5,5′-Dimethylluciferin (VIII).—The thiazoline synthesis referred to above also works with penicillamine

(3) S. Seto, K. Ogura, and Y. Nishiyama, *Bull. Chem. Soc. Japan*, **36**, 332 (1963).

(4) K. B. Wiberg and B. J. Nist, "Interpretation of N.m.r. Spectra," W. A. Benjamin, Inc., New York, N. Y., 1962.

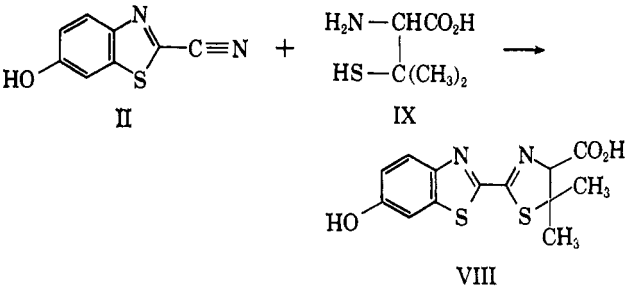
TABLE I

ULTRAVIOLET ABSORPTION OF FIREFLY LUCIFERIN ANALOGS

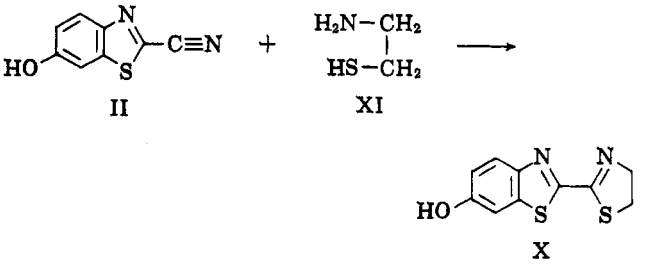
Compd.	Absorption bands, mμ (log ε)[a]	
XV		275 (3.59)
XIV	251 (3.78)	295 (4.22)
	247 (3.79)	
	243 (3.77)	
VII	268 (3.87)	326 (4.27)
X	261 sh	328 (4.24)
	268 (3.86)	
VIII	269 (3.84)	330 (4.24)
I	269 (3.85)	330 (4.26)

[a] In 95% ethanol.

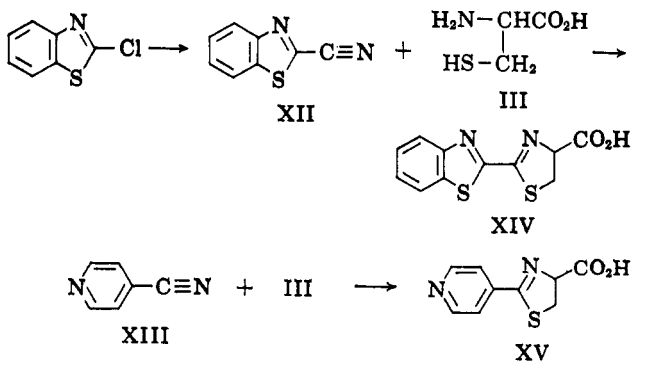
(IX) as shown by the synthesis of 2-(6′-hydroxy-2′-benzothiazolyl)-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (VIII). The proof of structure rests on the method of synthesis, the ultraviolet spectrum (Table I), the infrared spectrum, and the elementary analysis.



Decarboxyluciferin (X).—This compound was prepared by the condensation of hydroxynitrile II with cysteamine (XI). Similar condensations of cysteine



(III) with 2-cyanobenzothiazole (XII) and 4-cyanopyridine (XIII) yielded deshydroxyluciferin (XIV) and 2-(4′-pyridyl)-Δ^2-thiazoline-4-carboxylic acid (XV), respectively. The pyridyl compound XV is the only



luciferin analog that has been reported previously[5]; it had been prepared by a more circuitous route than ours.

(5) A. Banashek and M. N. Shchukina, *J. Gen. Chem. USSR* (Eng. Transl.), **30**, 3296 (1960).

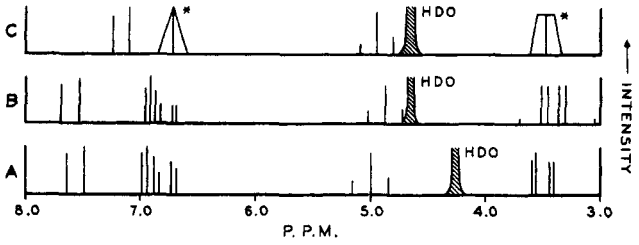
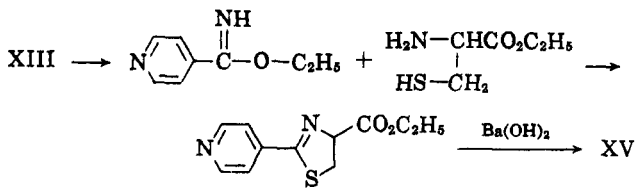


Figure 1.—The n.m.r. spectrum of firefly luciferin in D_2O + NaOD (external trimethylsilane = 0, τ = 10 − p.p.m.): A, 70°; B, 30° with excess base; C, 30°; *, not resolved.



The ultraviolet spectra for the various luciferins are given in Table I; compounds I, VII, VIII, and X have essentially identical spectra, as expected. The absorptions are a sensitive function of the substitution pattern as we will show later in a paper on the positional isomers of luciferin. The infrared spectra in KBr are all rich in detail; strong bands between 6.2 and 6.3, a broad band between 8 and 8.5, a strong band near 9.5, and one between 11 and 11.5 μ seem to be characteristic of the luciferin gross structure, however. The differences in the spectra of D-luciferin (I) and DL-luciferin (I) are also considerable; *e.g.*, the carbonyl band for the former compound is at 5.89 μ, whereas that for the latter is at 5.74 μ.

In addition to the analogs of luciferin reported in this paper, we have prepared three amino analogs, three positional isomers (OH group), and a hydroxyluciferin. In the entire group, only the 6-amino analog and 4′-hydroxyluciferin proved active in bioluminescence when tested with luciferase, ATP, magnesium ion, and oxygen, or when the luciferin–AMP anhydride (LH_2AMP) was treated with oxygen in the presence of luciferase. In the two positive cases, red light was emitted!

Experimental

Cysteine (III).—Cystine (0.218 g., 0.906 mmole) was dissolved in 50 ml. of liquid ammonia and reduced with sodium metal; small pieces were added until a blue color persisted for 10 min. The excess sodium was destroyed with ammonium chloride and the ammonia was removed with a stream of nitrogen. The dry residue was dissolved in 25 ml. of oxygen-free water (nitrogen bubbling is sufficient) and the pH was adjusted to 7.5 with hydrochloric acid. This solution was freshly prepared for each synthesis of luciferin.

2-Chloro-6-methoxybenzothiazole (VI).—This compound was prepared from 2-amino-6-methoxybenzothiazole (Aldrich Chemical Co., Inc.) by a slight modification of the procedure of Stuckwisch.[6] The crude material was extracted with cyclohexane rather than with ethanol; 35–45% yields of product melting at 50–52° were obtained. This material was chromatographed on alumina and sublimed to give crystals melting at 52.5–53°.

2-Chloro-6-hydroxybenzothiazole (V).—2-Chloro-6-methoxybenzothiazole (VI, 8.0 g., 40 mmoles) was heated with 60 g. of freshly prepared pyridine hydrochloride to 170° (bath temperature) for 3.5 hr. under magnetic stirring in a stoppered flask. The mixture was dissolved in water, and the solution was extracted three times with ether (300 ml.). The ether layer was dried, 10 g. of silica gel was added, and the solvent was removed *in vacuo*. The dry powder was placed on the top of a prepared column of silica gel and the desired compound was eluted with increasing amounts of ether in petroleum ether (b.p. 30–60°). Fractions having an ultraviolet absorption around 278 mμ (which was shifted to 317 mμ on addition of 1 drop of NaOH solution) were combined and rechromatographed on a column of SiO_2 using increasing amounts of ethyl acetate in petroleum ether as the eluent. The main fractions yielded 2-chloro-6-hydroxybenzothiazole with m.p. 175–176° (some sintering starting from 130°). The yield was 1.109 g. (5.97 mmoles, 14.9%). For analysis, part of this material was dissolved in a small volume of hot chloroform, and warm petroleum ether was added to turbidity. There resulted white crystals which, on heating, sublimed into transparent prisms at around 160° and melted at 175–176°.

Anal. Calcd. for C_7H_4ClNOS: C, 45.29, H, 2.18; Cl, 19.10; N, 7.55; S, 17.28. Found: C, 45.30; H, 2.25; Cl, 18.95; N, 7.45; S, 17.17.

Firefly Luciferin (I).—This compound was prepared from 2-cyano-6-hydroxybenzothiazole and cysteine by essentially the method published earlier.[1] Modifications in the procedure include the use of a slight excess of the nitrile, a reaction pH of 7.5, extraction of the basic reaction mixture with ethyl acetate to remove neutral compounds, and extraction of the acidified mixture with ethyl acetate. The acidification (with HCl) should be performed sufficiently slowly so that the liberated luciferin does not appear as a separate solid phase. The ethyl acetate solution from this treatment is dried and evaporated until the first crystals of luciferin appear; cooling then gives *ca.* 70% yields of luciferin in the form of white crystals which give only one spot on paper chromatography. All operations should be performed under nitrogen.

The n.m.r. spectrum of luciferin (as an anion) in D_2O is sensitive to the temperature of the solution and the amount of sodium hydroxide present. At 70° in the absence of an excess of base, the spectrum consists of an AMX series for the 4′-, 5′-, and 7′-protons and an example of an ABX series for the 4- and 5-protons which resembles an A_2X series (presumably as the result of a relatively large coupling between the A and B protons)[4]; the 4′-proton appeared as a doublet centered at τ 2.44 ($J_{4',5'}$ = 9.5 c.p.s.), the 7′-proton as a doublet centered at τ 3.035 ($J_{7',5'}$ = 2 c.p.s.), the 5′-proton as a pair of doublets centered at τ 3.21, the 4-proton as a symmetrical triplet centered at τ 5.00 ("J" = 9 c.p.s.), an HDO peak at τ 5.74, and the two 5-protons as a set of four peaks approximately equal in height at τ 6.41, 6.44, 6.56, and 6.595. This spectrum, that at 30°, and that at 30° in the presence of an excess of base are represented in Figure 1. At temperatures near 100°, the proton at position 4 is exchanged for deuterium in about 1 hr., and, on long exposures, the proton at position 7′ is also exchanged.

Radioactive Luciferin (I). A.—S^{35}-Luciferin was prepared from S^{35}-cysteine and 2-cyano-6-hydroxybenzothiazole by the methods outlined earlier.[1]

B.—C^{14}-Luciferin was prepared from 11.17 mg. (0.172 mmole) of potassium cyanide (3.6 mc./mmole) by the following procedure. The potassium cyanide was dissolved in 6 ml. of pure dimethyl sulfoxide by heating the mixture of compounds to 130–140° for 20 min. The temperature of the solution was lowered to 70° and 4 ml. of dry benzene was added. The mixture was boiled for 15 min. to remove most of the benzene and the remainder was removed *in vacuo*. 2-Chloro-6-methoxybenzothiazole (72 mg., 0.362 mmole) was added and the mixture was heated for 1 hr. at 140°. The red solution was cooled, diluted with ether (10 ml.), and partitioned between water (20 ml.) and ether (25 ml.). The aqueous phase was extracted a second time with ether (10 ml.) and the combined ether extracts were washed with water (10 ml.) and dried over sodium sulfate. The residue (73 mg.), on evaporation, was treated with chloroform, and alumina (0.5 g., Merck, acid-washed) was added. The chloroform was removed *in vacuo* and the residue was added to the top of a column prepared from 5 g. of acid-washed alumina and petroleum ether. The column was developed with petroleum ether containing increasing amounts of ethyl ether (up to 20%) until the main blue fluorescent band was eluted. All fractions with the characteristic ultraviolet absorption of the product (λ_{max} 320 mμ) were combined and the solvent was removed *in vacuo*. There was obtained 13 mg. (0.0684 mmole, 40%), of 2-(cyano-C^{14})-6-methoxybenzothiazole (IV).

(6) C. G. Stuckwisch, *J. Am. Chem. Soc.*, **71**, 3417 (1949); see J. Metzger and H. Plank, *Bull. soc. chim. France*, 1701 (1956), for a similar preparation.

In a similar experiment, unlabeled potassium cyanide (16.4 mg., 0.252 mmole) was reacted as described above with 2-chloro-6-methoxybenzothiazole (75 mg., 0.376 mmole). After chromatography on alumina (Merck, acid-washed) there was obtained 35.0 mg. (0.184 mmole, 73%) of 2-cyano-6-methoxybenzothiazole, m.p. 129–130° (lit.[1] m.p. 129–131°). The *labeled* 2-(cyano-C^{14})-6-methoxybenzothiazole (13 mg.) was diluted with unlabeled material (17 mg.), giving a total of 30.0 mg. (0.158 mmole) of 2-cyano-6-methoxybenzothiazole. This mixture was dissolved in a small amount of chloroform and then transferred to a tube of small diameter containing a small bulb at the bottom with a capacity of *ca.* 1 ml. The chloroform was evaporated at 40° with a slow stream of nitrogen. Pyridine hydrochloride (0.41 g., 3.55 mmoles) was introduced, using a funnel, and the tube was sealed *in vacuo*. The mixture was heated for 1 hr. at a temperature of 170–190° in an oil bath. After cooling, the reaction mixture was partitioned between water (10 ml.) and ethyl acetate (20 ml.). The extraction with ethyl acetate was repeated once more. The residue (26 mg.) left on evaporation of the ethyl acetate was dissolved in methanol and the solution was evaporated to dryness on 0.3 g. of Davidson 923 silica gel; this material was then added to the top of a column of 5 g. of silica gel in petroleum ether. The greenish blue fluorescent main band was eluted with increasing concentrations (0 to 30%) of ether in petroleum ether (Phillips Petroleum Co.). All fractions showing an ultraviolet absorption at 323 mμ (shifted to 386 mμ on addition of a small droplet of dilute potassium hydroxide) were combined and evaporated to dryness. The yield was 9.0 mg. (0.05 mmole, 32.3%) of 2-(cyano-C^{14})-6-hydroxybenzothiazole (II).

In a similar experiment with unlabeled 2-cyano-6-methoxybenzothiazole (35 mg, 0.184 mmole), there was obtained 26.5 mg. (0.155 mmole, 81.6%) of 2-cyano-6-hydroxybenzothiazole, m.p. 203–206° (lit.[1] m.p. 212–215°).

D-Cystine (10 mg., 0.042 mmole) was reduced as described above, and the cysteine was dissolved in 1 ml. of water. The labeled 2-cyano-6-hydroxybenzothiazole (9 mg., 0.051 mmole), dissolved in methanol (1 ml.), was added and the mixture was allowed to stand for 1 hr. in the dark (the water and methanol had been saturated with nitrogen; pH 8.5). The solution was extracted with ethyl acetate, diluted with water (4 ml.), and acidified with 1 *N* hydrochloric acid (0.2 ml.). The product was collected after cooling overnight in the freezer. There was obtained 11 mg. (0.0393 mmole, 77%) of C^{14}-luciferin (I). The identity and purity of this material was proved by comparing the paper chromatograms and light yields in the enzymatic reaction with similar data obtained with unlabeled, analytically pure D-luciferin.

C. Alternative Synthesis of C^{14}-Luciferin.—Potassium cyanide (4.4 mg., 0.068 mmole) was dissolved in 4 ml. of dimethyl sulfoxide by heating and stirring the mixture in an oil bath (bath temperature 150°) for 15 min. in a stoppered flask. To the clear solution, 2-chloro-6-hydroxybenzothiazole (11.0 mg., 0.059 mmole) was added and the resulting solution was heated and stirred in the stoppered flask for 2 hr. (bath temperature 140–150°). The mixture was then partitioned between water (40 ml.) and ether (40 ml.). The ether extraction was repeated a second time using an additional 20 ml. of ether. The combined ether layers were washed with water and dried over sodium sulfate, and the ether was evaporated The residue was chromatographed on a SiO_2 column using ether–petroleum ether mixtures to elute the product. The appropriate fractions were combined to give, on evaporation, 2-cyano-6-hydroxybenzothiazole (6.85 mg., 0.039 mmole, 66%), m.p. 205–209° (lit.[1] m.p. 212–215°).

The synthesis was repeated using a mixture of 16 mg. of $KC^{14}N$, 16 mg. of unlabeled KCN (0.49 mmole), and 120 mg. of the chloride (0.65 mmole). The product was chromatographed two times on SiO_2 columns. There was obtained 19.7 mg. (0.11 mmole, 24%) of 2-cyano-6-hydroxybenzothiazole, which was converted into luciferin by the method outlined in part B.

2-Cyano-6-methoxybenzothiazole (IV).—This compound was prepared originally from *p*-anisidine.[1] It is more conveniently prepared now from 2-chloro-6-methoxybenzothiazole (VI) as described in the synthesis of radioactive luciferin. A second convenient synthesis has been reported by Japanese workers.[3]

2-(6′-Methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic Acid (VII).—Cysteine was prepared from 0.44 g. (1.81 mmole) of DL-cystine, as described above, and dissolved in 50 ml. of water. Methanol (50 ml.) was added and then 0.76 g. (3.99 mmole) of 2-cyano-6-methoxybenzothiazole dissolved in 70 ml. of methanol was added. The mixture was kept at 25° for 3 hr. and then filtered from a small amount of precipitate. The methanol was removed *in vacuo* leaving a mixture of water and luciferin; the precipitate was dissolved by the addition of 0.1 *N* sodium hydroxide to a pH of 8.5. Water (60 ml.) was added and the solution was extracted with ethyl acetate, then acidified with hydrochloric acid to pH 1. The white precipitate was collected, washed with water, and dried *in vacuo*. There was obtained 0.96 g. (3.26 mmoles, 89%) of 2-(6′-methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (VII), m.p. 172–174° dec. Recrystallization of this sample from acetone and then from an acetone–cyclohexane mixture in dim light under nitrogen yielded white needles, m.p. 176–177° dec. See Table I for the ultraviolet absorption. The n.m.r. spectrum of a saturated solution was measured in perdeuterioacetone at 65° (see discussion section).

Anal. Calcd. for $C_{12}H_{10}N_2O_3S_2$: C, 48.95; H, 3.42; N, 9.52; S, 21.79. Found: C, 48.85; H, 3.46; N, 9.31; S, 22.00.

2-(6′-Hydroxy-2′-benzothiazolyl)-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic Acid (VIII).—2-Cyano-6-hydroxybenzothiazole (0.36 g., 2.04 mmoles) was dissolved in 15 ml. of methanol in a flask kept flushed with nitrogen, and a solution of DL-penicillamine (0.34 g., 2.28 mmoles) in 5 ml. of water (adjusted to pH 8 with sodium hydroxide) was added. Aliquots were removed and acidified and their ultraviolet absorptions were determined. An absorption of 324 mμ was observed at the start of the reaction; at the end (after *ca.* 90 min.) the absorption was 332 mμ. The solution was diluted with 70 ml. of water and the pH was adjusted to 8.5. The solution was extracted with ether and then with ethyl acetate to remove the neutral compounds and then it was acidified with 10% HCl to pH 1. The yellow precipitate was collected, washed with water, and dried *in vacuo*. The yield was 0.57 g. (1.85 mmoles, 90.5%) of 5,5-dimethyluciferin (VIII), m.p. 191–193° dec.

The analytical sample was prepared by two recrystallizations from an acetone–cyclohexane mixture, m.p. 194–196° dec. See Table I for the ultraviolet absorption.

Anal. Calcd. for $C_{13}H_{12}N_2O_3S_2$: C, 50.64; H, 3.90; N, 9.09; S, 20.80. Found: C, 50.78; H, 4.04; N, 9.08; S, 21.01.

Recrystallization of the crude material from aqueous methanol yielded crystals, m.p. 188–190° dec., which gave an analysis corresponding to the dihydrate.

Decarboxyluciferin (X).—A solution of 2-cyano-6-hydroxybenzothiazole (1.0 g., 5.7 mmoles) and cysteamine (1.0 g., 13 mmoles) in absolute ethanol (100 ml.) was refluxed under nitrogen for 18 hr. The solid which separated on cooling (1.23 g.) was collected and recrystallized from absolute ethanol to give 1.02 g. (4.3 mmoles, 76%) of 2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline (X), m.p. 283–292° dec. A sample for analysis was prepared by two more recrystallizations from ethanol followed by sublimation *in vacuo* at 200°, m.p. 289–292°. See Table I for the ultraviolet spectrum.

Anal. Calcd. for $C_{10}H_8N_2OS_2$: C, 50.83; H, 3.41; N, 11.86; S, 27.14. Found: C, 50.96; H, 3.55; N, 11.92; S, 27.37.

2-(2′-Benzothiazolyl)-Δ^2-thiazoline-4-carboxylic Acid (XIV).

A. 2-Cyanobenzothiazole (XII).—Potassium cyanide (2.5 g., 38.4 mmoles) was suspended in 400 ml. of dimethyl sulfoxide and 50 ml. of benzene, and the mixture was heated until about 5 ml. of benzene had distilled. 2-Chlorobenzothiazole (4.67 g., 27.6 mmoles) was added and heating was continued at 120–128° until the peak at 254 mμ in the ultraviolet spectrum of an aliquot had been replaced by the 287-mμ peak of the product (about 30 min. was required). The reaction mixture was cooled and then added to 400 ml. of ice and water. The pH was adjusted to 5.0 and the solution was transferred to a separatory funnel. The water layer was removed and about 250 ml. of ether was added to the benzene. The benzene–ether solution was washed three times with water (50 ml.) and then dried over sodium sulfate. The water layer was extracted eight times with 200 ml. of ether. The ether layer was washed three times with 100 ml. of water and dried over sodium sulfate. The organic extracts were combined and evaporated to dryness and the resulting crude 2-cyanobenzothiazole was chromatographed. The solid was dissolved in a minimum quantity of chloroform (about 75 ml.) and 35 g. of acid-washed alumina (Merck) was added to the solution. The chloroform was removed *in vacuo* and the alumina was added to the top of a column containing 350 g. of acid-washed alumina in petroleum ether. The 2-cyanobenzothiazole was eluted with petroleum ether containing increasing amounts of ether. A total of 10 l. of petroleum ether and 4 l. of ether was used; 1-l. fractions were

collected. Fractions 8 to 10 contained a small amount of starting material, whereas fractions 11 to 14 contained the product. The melting points of the material in fractions 12, 13, and 14 were 75–76, 78, and 74–76°, respectively. Recrystallization from chloroform, to which isooctane was added, yielded 2.96 g. (18.5 mmoles, 67%) of the nitrile XII, m.p. 78–80°. Sublimation at 60–70° under high vacuum increased the melting point to 79.5–80.5°, λ_{max} 287 mμ (ε 8.5 × 10³) and 244 mμ (ε 7.8 × 10³).

Anal. Calcd. for $C_8H_4N_2S$: C, 59.98; H, 2.52; N, 17.49; S, 20.02. Found: C, 59.90; H, 2.57; N, 17.49; S, 20.09.

B. Deshydroxyluciferin (XIV).—DL-Cysteine was prepared by the reduction of cystine (0.26 g., 1.08 mmoles) as described above; it was dissolved in 25 ml. of water and then adjusted to pH 7.5. Methanol (25 ml.) and then a solution of 2-cyanobenzothiazole (0.37 g., 2.31 mmoles) in 25 ml. of methanol was added. The mixture was allowed to stand for 85 min. during which time the ultraviolet absorption shifted from 288 to 292 mμ. Water was added (40 ml.) and the methanol was evaporated *in vacuo*. The pH of the resulting solution was adjusted to about 9 by addition of 10% sodium hydroxide, and the mixture was extracted with ethyl acetate (150 ml.). The aqueous solution was filtered and acidified with hydrochloric acid to pH 1. The whitish precipitate was collected, washed with water, and dried *in vacuo* at 65°. There was obtained 0.50 g. (1.92 mmoles, 89%) of 2-(2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid (XIV), m.p. 164–166°. For analysis, this material was recrystallized from methanol, m.p. 166–167°. See Table I for the ultraviolet absorption.

Anal. Calcd. for $C_{11}H_8N_2O_2S_2$: C, 49.98; H, 3.05; N, 10.60; S, 24.26. Found: C, 50.40; H, 3.10; N, 10.43; S, 24.17.

2-(4′-Pyridyl)-Δ²-thiazoline-4-carboxylic Acid (XV).—Cysteine was prepared by the reduction of DL-cystine (0.73 g., 3.35 mmoles) as described above; it was dissolved in 20 ml. of water and adjusted to a pH of about 8. Methanol (20 ml.) was then added, followed by a solution of 4-cyanopyridine (0.57 g., 4.06 mmoles) in 20 ml. of methanol. The mixture was allowed to react overnight. Most of the methanol was removed *in vacuo*, water was added (80 ml.), the pH was adjusted to 8.5, and the mixture was extracted with ethyl acetate (250 ml.). The aqueous phase was filtered, the dissolved ethyl acetate was removed in a stream of nitrogen, and the pH was lowered to about 2 by the addition of hydrochloric acid. The precipitate was collected, washed with water, and dried *in vacuo* to give 0.74 g. (3.55 mmoles, 58.5%) of 2-(4′-pyridyl)-Δ²-thiazoline-4-carboxylic acid (XV), m.p. 177–179° dec. For analysis, the compound was crystallized from methanol under nitrogen in dim light, m.p. 178–179.5° dec. At a slower rate of heating, the melting point was observed at 173–174° dec. (lit.[5] m.p. 174–175°). See Table I for the ultraviolet absorption.

Anal. Calcd. for $C_9H_8N_2O_2S$: C, 51.91; H, 3.87; N, 13.45; S, 15.40. Found: C, 52.07, 51.88; H, 3.99, 3.95; N, 13.49, 13.45; S, 15.44, 15.49.

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Spectroscopic Studies on Dyes. V. Derivatives of *cis*-Indigo[1]

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A comparison of the visible spectrum of N,N′-oxalylindigo with the spectra of the photochemically produced *cis* isomers of N,N′-diacetylindigo and N,N′-bis(trifluoroacetyl)indigo suggests a noncoplanar *cis* configuration for all three molecules. This was further substantiated by a study of the carbonyl stretching frequencies in the infrared region. The formation of oxalylindigo from indigo and oxalyl chloride was studied in benzene and chlorobenzene solutions, using spectrophotometric techniques. The results indicate a two-step mechanism with *trans*-N-(chlorooxalyl)indigo as the intermediate. By a similar technique the alkaline hydrolysis of oxalylindigo in aqueous dimethylformamide was found to proceed in two steps involving the stepwise hydrolysis of the two amide linkages. Owing to the slowness of the second step it was possible to determine the visible spectrum of the intermediate anion and to assign a *trans* configuration to it in view of the long wave length (571 mμ) of the absorption maximum. (Heller had previously observed a blue intermediate in this reaction which he attributed to *cis*-indigo.)

Although the structure of indigo has long been known and it is recognized that the molecule possesses a *trans* configuration,[3,4] derivatives of *cis*-indigo have been reported in the literature[5] and the existence of *cis*-indigo has also been postulated.[6] In view of our current understanding of the *cis–trans* isomerization of conjugated compounds, it is considered highly unlikely that the unstable *cis* isomer could exist at ambient temperatures (except, possibly for a few microseconds).[4,7] Recent theoretical and spectroscopic studies aimed at a better understanding of the indigo chromophore system[8] are also in accord with this conclusion.

This investigation was undertaken with the objective of clarifying the structure of N,N′-oxalylindigo, which had been variously reported as having a *trans*[6] and a *cis*[4] configuration. Since in the first part of this work we concluded that this compound exists in the *cis* configuration (it is, indeed, the only derivative of *cis*-indigo that can be made directly from indigo!), it was considered desirable to study the mechanism of its formation. In addition, some of the evidence reported by Heller[6] for the formation of "*cis*-indigo" during the alkaline hydrolysis of oxalylindigo was re-examined.

Experimental

Absorption spectra for the substituted indigos are shown in Figures 1–3. Figure 4 shows the spatial arrangement of the carbonyl groups in a *cis*-indigo.

N,N′-Diacetylindigo was prepared by the method of Liebermann and Dickhuth.[9]

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(3) T. Posner, *Ber.*, **B59**, 1799 (1926).

(4) W. R. Brode, E. G. Pearson, and G. M. Wyman, *J. Am. Chem. Soc.*, **76**, 1034 (1954).

(5) (a) R. Pummerer and F. Meininger, *Ann.*, **590**, 173 (1954); P. Friedländer and L. Sander, *Ber.*, **B57**, 637 (1924).

(6) G. Heller, *ibid.*, **B77**, 163 (1944).

(7) (a) G. M. Wyman, *Chem. Rev.*, **55**, 625 (1955); (b) E. Fischer and Y. Frei, *J. Chem. Phys.*, **27**, 808 (1957); (c) G. Gabor and E. Fischer, *J. Phys. Chem.*, **66**, 2478 (1962).

(8) (a) W. Lüttke and M. Klessinger, *Chem. Ber.*, **97**, 2342 (1964); (b) M. Klessinger and W. Lüttke, *Tetrahedron*, **19**, 315 (1963).

(9) C. Liebermann and F. Dickhuth, *Ber.*, **24**, 4131 (1891).