Tetrahedron. Vol. 28, pp. 4065 to 4074. Pergamon Press 1972. Printed in Great Britain

STUDIES ON FIREFLY BIOLUMINESCENCE—I

SYNTHESIS AND SPECTRAL PROPERTIES OF FIREFLY OXYLUCIFERIN,* A POSSIBLE EMITTING SPECIES IN FIREFLY BIOLUMINESCENCE†

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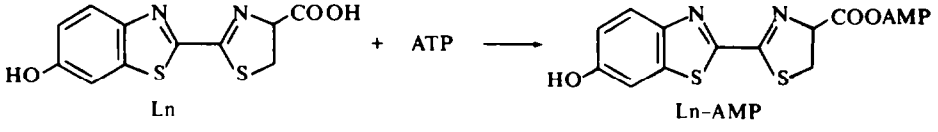
(*Received in Japan* 3 *April* 1972; *Received in the UK for publication* 2 *May* 1972)

Abstract— Oxyluciferin, 2-(6'-hydroxybenzothiazol-2'-yl)-4-hydroxythiazole (I), a proposed emitter in firefly bioluminescence, was synthesized and identified as the emitter in firefly chemiluminescence. It was also shown that oxyluciferin (I) can be converted chemically to firefly luciferin.

DURING the last decade considerable progress has been made in our understanding of the firefly bioluminescence mechanism.[1] Firefly bioluminescence is produced by the enzymatic oxidation of firefly luciferyl adenylate (Ln–AMP), which is formed by reaction of luciferin (Ln) and ATP in the presence of magnesium ion, luciferase and 1 mole of O_2 resulting in CO_2, AMP and product.[2–4]



Ln Ln–AMP

$$\text{Ln–AMP} + O_2 \rightarrow \text{Product} + CO_2 + \text{AMP} + h\nu$$

Luciferyl adenylate was found to be strongly chemiluminescent in basic DMSO solution,[2, 5, 6] and the chemiluminescence mechanism has been studied intensively. Luciferin is easily oxidized without light emission to dehydroluciferin,[7] making analysis of the light-producing process confusing, thus the chemiluminescence mechanism has been studied using 5,5-dimethyl derivatives of luciferin,[2, 3, 6] which have no oxidizable hydrogen atoms at the 5-position. McCapra *et al.*[3] reported that the phenyl ester of 5,5-dimethylluciferin (IIa) in DMSO, when treated with guanidine carbonate, gave a red chemiluminescence and that the emitter could be 5,5-dimethyl-decarboxyketoluciferin (5,5-dimethyl-oxyluciferin) (III), which was isolated from the reaction. The same red chemiluminescence was observed by Seliger and White[2, 6] with luciferyl adenylate as well as 5,5-dimethylluciferyl adenylate (IIb). They prepared III and showed that the fluorescence emission spectrum of the anion of III in DMSO is identical with the chemiluminescence spectrum of IIb, thus confirming the emitter of the red colour to be the excited state of the monoanion of III.

* 2-(6'-Hydroxybenzothiazol-2'-yl)-4-hydroxythiazole. The name "firefly decarboxyketoluciferin" has appeared in the literature,[1] but we propose the name "firefly oxyluciferin" from analogy with other bioluminescent systems.[1,4]

† Preliminary communication, *Tetrahedron Letters* 4683 (1969)

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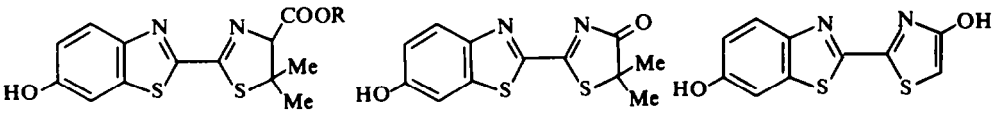


IIa: R = Ph, IIb: R = AMP III I

From these results and other indirect evidence, both groups suggested a chemiluminescence mechanism involving a dioxetane intermediate as shown in Fig 1.

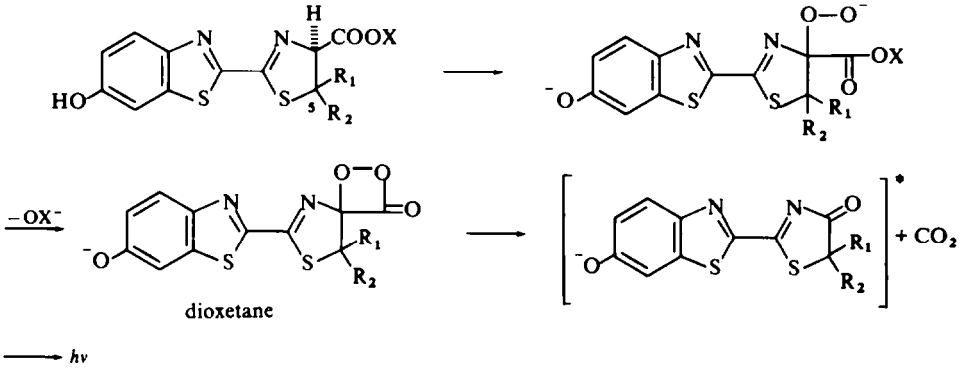
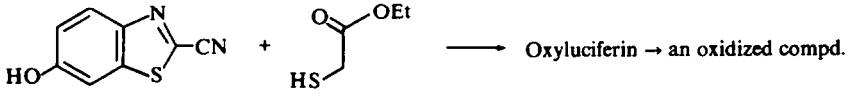


FIG 1. A proposed firefly chemiluminescence mechanism

The colour of normal firefly bioluminescence *in-vitro* at pH 7·6 is yellow-green, but under acidic conditions, e.g. pH 5·6, red bioluminescence is observed.[8,9] In the case of chemiluminescence of luciferin derivatives, the colour is also dependent on base concentration.[2,6] White *et al.*[6] reported that the phenyl ester of luciferin (IIa), when treated with small amounts of t-BuOK in DMSO, yielded red chemiluminescence but large amounts of base lead to a yellow-green emission. These two different emissions correspond closely to the two colours of bioluminescence, and they attributed the emitter of the yellow-green luminescence to the excited state of the dianion of I. However, attempted isolation or identification of the emitter, firefly oxyluciferin (I), from the solutions of chemi- and bioluminescence reactions has been unsuccessful,[10] and instead three coloured products were isolated. These three products were also produced when attempted synthesis of oxyluciferin (I) was carried out by condensation of ethyl thioglycolate and 2-cyano-6-hydroxybenzothiazole (X), and hence White *et al.*[10] concluded that oxyluciferin (I) is the immediate product, but was very unstable and degraded into three compounds. Re-investigating their synthetic method in consideration of the reaction mechanism, we were able to synthesize oxyluciferin (I), the proposed emitter of firefly luminescence.



X I VII

Among the methods for the synthesis of 5-unsubstituted 2-aryl-4-thiazolones reported,[11,12] the method of condensation of a nitrile and a thioglycolate would only

be sufficiently mild to synthesize unstable compounds. Applying this method White *et al.* synthesized 5,5-dimethyl- (III) and 6'-O-methyl-oxyluciferin,[2,6] but as mentioned attempted synthesis of oxyluciferin (I) failed.

We found that although derivatives of oxyluciferin such as 5,5-dimethyl- (III), 5-methyl- (IV), 6'-O-methyl- (V),[6,13] and 6'-O-acetyl-oxyluciferin (VI)[13] could be synthesized from the appropriate nitriles and thioglycolic esters in the usual way, (*ca* 50% aq. MeOH at pH 8, few hr, room temp), attempted condensation of ethyl thioglycolate and nitrile X under similar conditions did not give the desired compound, oxyluciferin (I), but dioxyluciferin (VII) containing an extra oxygen atom as shown by elemental analysis and its mass spectrum (Fig 2). The IR spectrum of this compound showed a broad strong band at 1680 cm^{-1} indicating the presence of a conjugated carbonyl group (Fig 3). That the benzothiazolylthiazole ring system is retained in this molecule is indicated by its UV spectrum (Fig 4).

Broad NMR signals suggest that this compound exists as a tautomeric mixture, but also the presence of a 1,2,4-trisubstituted benzene ring and a methine proton (4·0 ppm) indicating the 5-position has been oxidized (Fig 5).

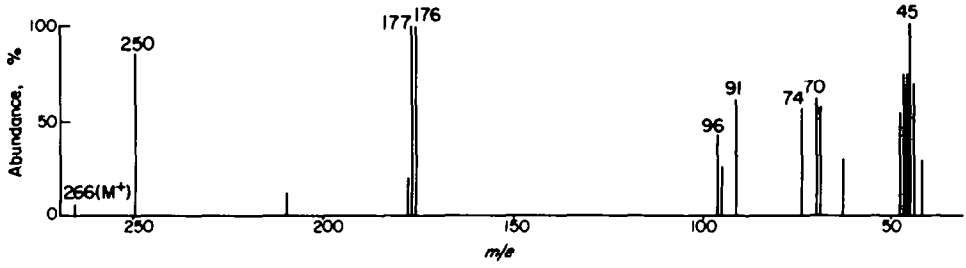


FIG 2. Mass spectrum of dioxyluciferin (VII)

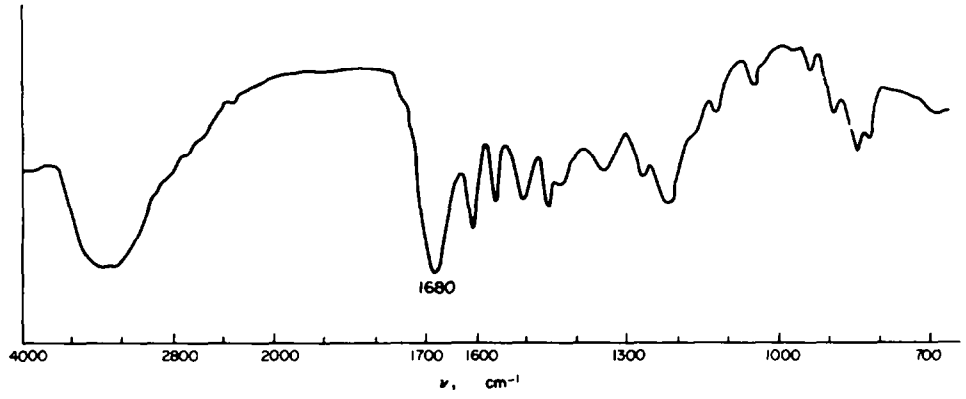


FIG 3. IR spectrum of dioxyluciferin (VII)

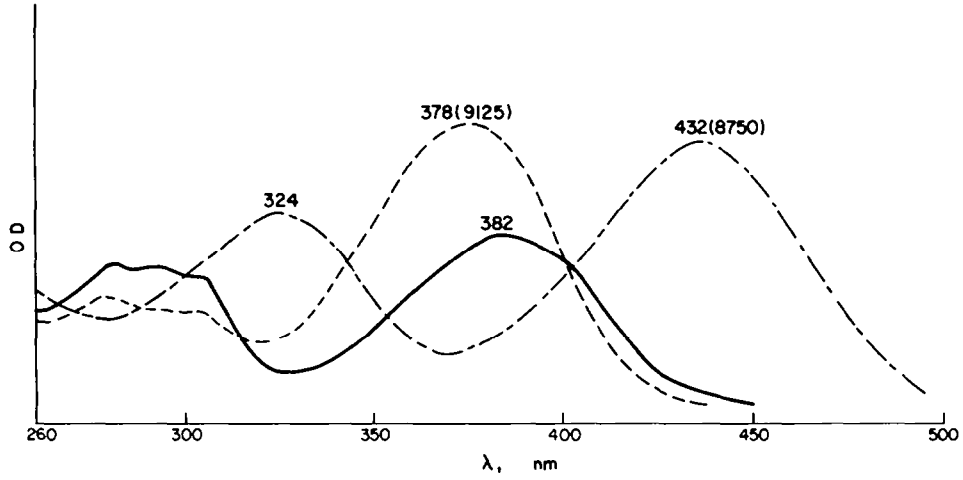


FIG 4. UV spectra of dioxyluciferin (VII): (MeOH) ———; (MeOH–HCl) – – – –; (MeOH–KOH) –·–·–

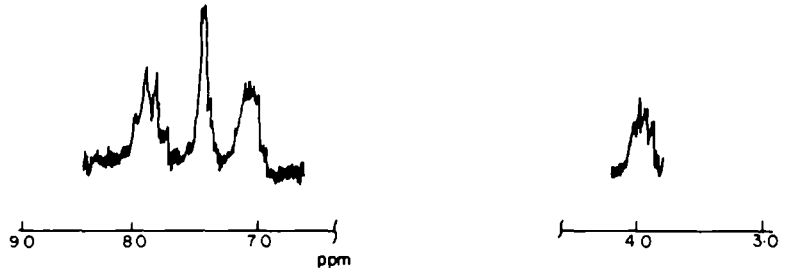
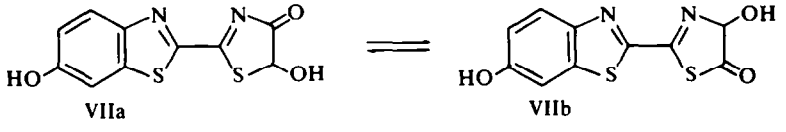


FIG 5. NMR spectrum of dioxyluciferin (VII) (DMSO-d_6)



VIIa VIIb

Dioxyluciferin VII

Acetylation of this compound gave a diacetate, whose NMR spectrum suggests that this compound is a mixture of diacetates, VIIIa and VIIIb (Fig 6 and 7).

The observation that the reaction times necessary for production of the 5-methyl derivative (IV) is *ca.* 10 times shorter than that for the 5,5-dimethyl derivative (III) suggests that condensation of unsubstituted thioglycolate with the nitrile would proceed much faster than in the case of the 5-methyl derivative (IV). Since instability of oxyluciferin (I) would be due to susceptibility towards oxygen, synthesis of oxyluciferin (I) should be conducted rapidly with exclusion of oxygen. Indeed, oxyluciferin (I) was obtained when condensation of thioglycolate and the nitrile was carried out in MeOH for 2 min. with ice-cooling under N_2.

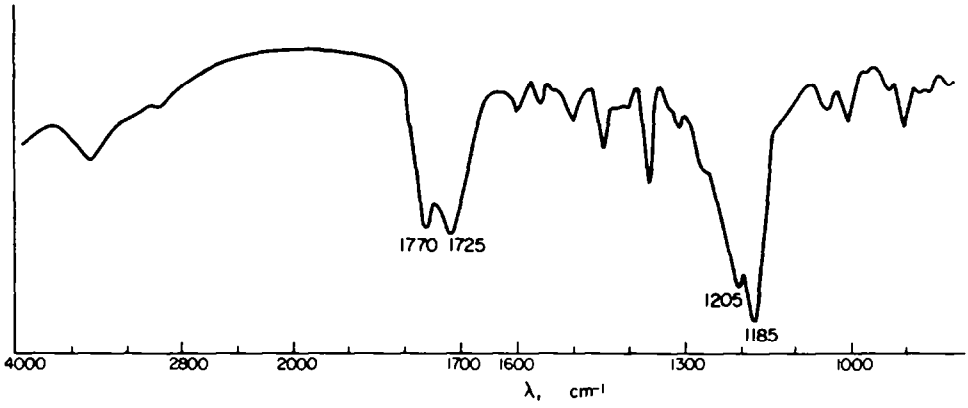


FIG 6. IR spectrum of dioxyluciferin diacetate (VIII) (KBr)

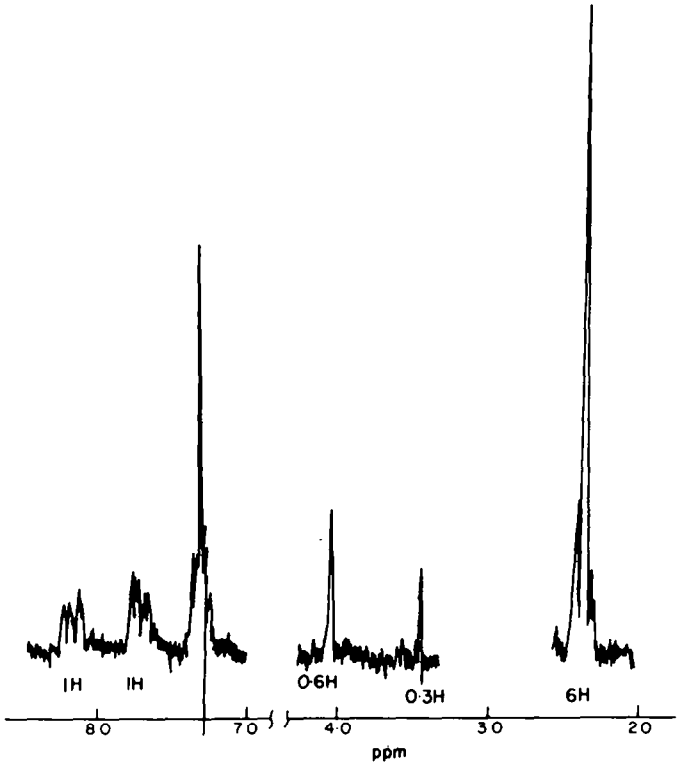


FIG 7. NMR spectrum of dioxyluciferin diacetate (VIII) ($CDCl_3$)

The orange-yellow crystalline powder thus obtained was sufficiently pure for elemental analysis but when recrystallized became rather impure. Elemental analysis and the mass spectrum (Fig 8) showed its molecular formula to be $C_{10}H_6N_2O_2S_2$, and its NMR and IR spectra confirmed structure I (Fig 9 and 10). The sharp singlet at 6·52 ppm indicates the presence of an olefinic proton at C_5, and hence oxyluciferin

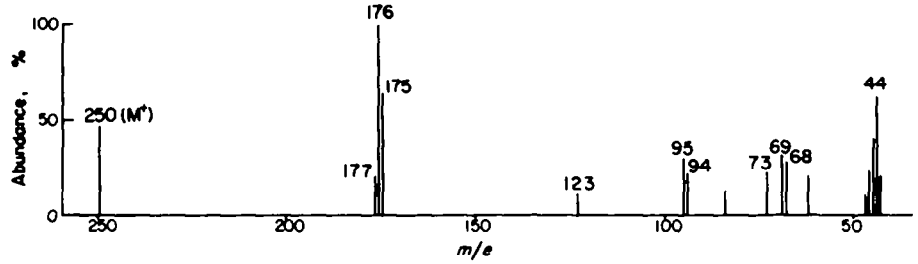


FIG 8. Mass spectrum of oxyluciferin (I)

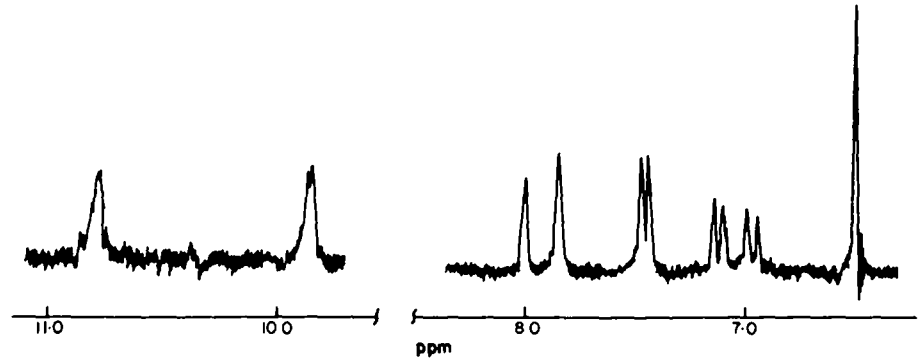


FIG 9. NMR spectrum of oxyluciferin (I) (DMSO-d_6)

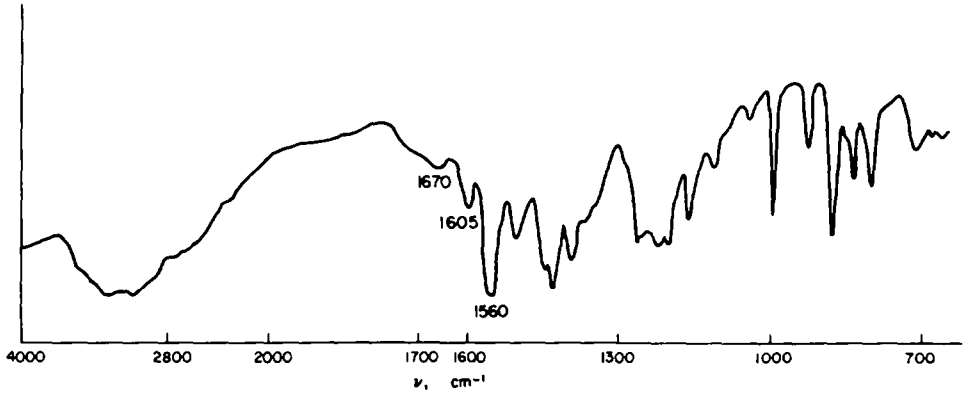


FIG 10. IR spectrum of oxyluciferin (I) (KBr)

(I) exists in DMSO as the enol-form, and likewise in acetone. The pKa's of I measured by the UV method are 6·85 and 8·60,[13] the former being attributable to the enolic hydroxyl group and the latter to the phenolic hydroxyl group.

A DMSO solution of oxyluciferin (I) was mixed with a DMSO solution of guanidine carbonate under vacuum (10^{-5} mm Hg) to give a red solution, whose fluorescence

spectrum showed its maximum at 556 ± 3 nm (FWHM 2300 cm^{-1} ± 10%). This fluorescence spectrum is almost superimposable with the spectrum of the yellow-green chemiluminescence (max 555 nm, FWHM 2050 cm^{-1} ± 10%) of luciferin reported by White *et al.*,[6] supporting the assignment of the dianion of I as the emitter of the yellow-green chemiluminescence. When mixing in the presence of air, the resulting solution is yellow and shows a fluorescence maximum at 498 ± 3 nm (FWHM 2550 cm^{-1})* (Fig 11).

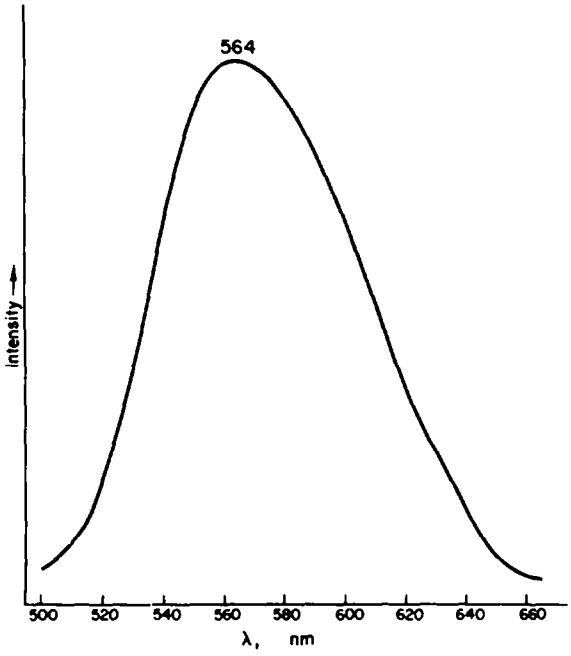
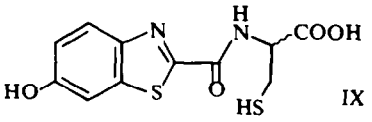


FIG 11. Fluorescence spectrum of oxyluciferin (I): 0·2 μmole of I in 2 ml of DMSO + 600 μl of 0·05N guanidine carbonate

In firefly lanterns the final product of luminescence, oxyluciferin (I), might be used for re-synthesis of luciferin and, hence, chemical transformation of oxyluciferin (I) to luciferin was investigated.

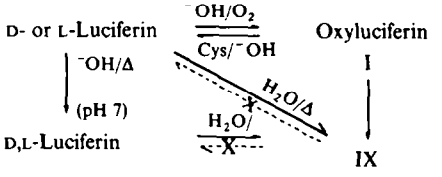
Oxyluciferin (I), when heated with cysteine in basic media, gave luciferin which was identified by comparison of the R_f value on TLC and the UV spectrum with those of authentic sample. Another fluorescent compound was produced as by product, which was also obtained from luciferin by hydrolysis with water and its structure was assigned as IX from the following data. The molecular formula is estimated from elemental analysis and mass spectrum. The UV spectrum of IX is superimposable with that of 6-hydroxybenzothiazolyl-2-carboxamide. The presence of an amide and a carboxyl group is suggested from the IR spectrum, whereas the NMR spectrum indicates the presence of the grouping $HX—\underset{|}{CH}—CH_2—Y$, where X, and Y are hetero atoms. A negative ninhydrin test excludes the alternative structure having a free amino group.

* FWHM = full band width between half-maximum intensity points of the spectrum.



IX

No optical activity was observed on IX, even though optically active starting material was used. It is known that optically active luciferin is racemized by heating with 1N NaOH to give racemic luciferin (D,L-luciferin), but the possibility of IX as an intermediate of recemization is eliminated since the amide (IX) could not be cyclized to luciferin by heating in aqueous solutions at any pH.



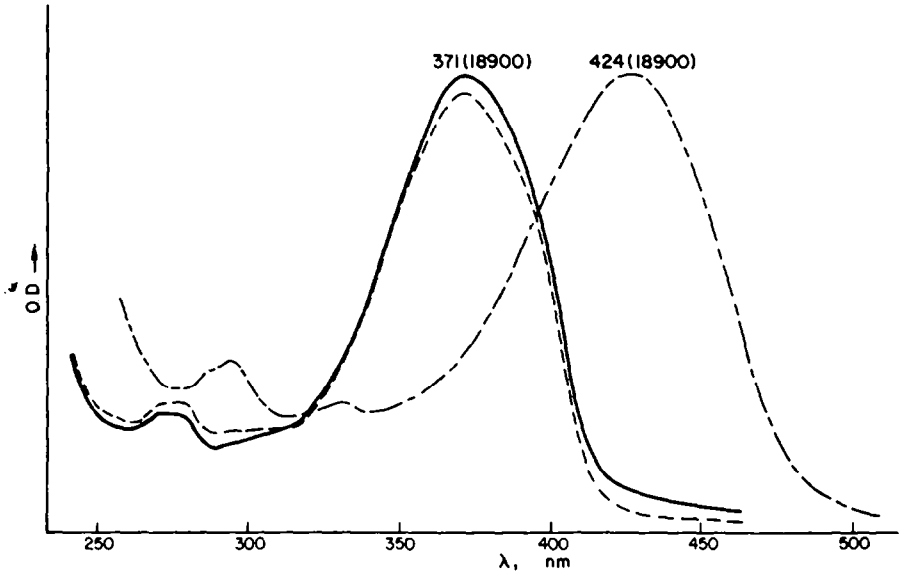


FIG 12. UV spectra of oxyluciferin (I): (MeOH) ———; (MeOH–HCl) – – – –; (MeOH–KOH) –·–·–

EXPERIMENTAL

All m.ps are uncorrected. The following spectrometers were used: IR: Jasco IR-E; UV: Hitachi EPS-3T; NMR: Jeol JNM-4H-100 (100 MHz), JNM-MH-100 (100 MHz) and Varian A-60 (60 MHz); MS: Hitachi RMU-6D; and fluorescence spectra: Hitachi MPF-3. Chemical shifts (δ) are in ppm from int. TMS and coupling constants in Hz (accuracy $\pm 0{\cdot}3$ Hz).

L- and D,L-luciferin were synthesized according to the methods described by White *et al.*[7] L-Luciferin. Yellow needles, m.p. 200–203° (lit.[7] 200–202°). UV (MeOH) 269·5 (8920), 434 (21,900); MeOH–HCl) 269, 332; (MeOH–KOH) 285, 385. D,L-Luciferin. (a) From D,L-cysteine. Yellow-brown crystalline powder, m.p. 198–199° (dec). (Found: C, 47·52; H, 3·00; N, 10·03. $C_{11}H_8N_2O_3S_2$ requires: C, 47·13; H, 2·86; N, 10·00%). IR (KBr) 3100 (br), 1745, 1610, 1565.

(b) From L-luciferin. A suspension of L-luciferin (0·30 g) in water (2·0 ml), adjusted to pH 7 with 1N NaOH aq and sealed in glass tubing under reduced pressure (water aspirator), was heated at 110° for 2 hr. The reddish-brown mixture was filtered and the filtrate acidified with dil. HCl. The resultant ppt was crystallized from MeOH aq to give pale-yellow needles, m.p. 202° (0·30 g). The IR spectrum was superimposable on that from D,L-cysteine. This compound was also obtained when L-luciferin (0·4 g) was dissolved in 1N NaOH aq and treated as above; yellow needles, m.p. 192–194° (0·16 g), identified by IR spectrum.

2-(6'-*Hydroxybenzothiazol-2'-yl*)-5,5-*dimethyl*-4-*thiazolinone*, (III). Although synthesis of this compound was announced by McCapra *et al.*[3] and by Hopkins *et al.*,[2] no data other than some fluorescence spectra were given, and hence our data are presented.

A solution of 2-cyano-6-hydroxybenzothiazole (X) (56 mg) in MeOH aq (50%, 10 ml) was added to a solution of ethyl mercaptoisobutyrate (75 μl) in MeOH (1 ml) under N_2 and the mixture adjusted to pH 8 by 1N NaOH aq. After stirring for 5 hr, the mixture was acidified with dil. HCl and the ppt collected. Crystallization from a mixture of DMSO, MeOH, and water gave yellow flat needles, m.p. 271–8° (dec). (Found: C, 51·71; H, 3·49; N, 9·79. $C_{12}H_{10}N_2O_2S_2$ requires: C, 51·78; H, 3·62; N, 10·06%). IR (KBr) 1725, 1605, 1495; UV (MeOH) 388 (23,500), 277 (6570); (MeOH–HCl) 390 (5050); (MeOH–KOH) 489 (7480); NMR (DMSO-d_6) 1·68 (6H, s), 7·18 (1H, d, d, J = 2·5, 9·0), 7·58 (1H, d, J = 2·5), 8·13 (1H, d, J = 9·0); *m/e* 278 (M^+).

2-(6'-*Hydroxybenzothiazol-2'-yl*)-4-*hydroxy*-5-*methylthiazole* (IV). From X. A solution of the nitrile (X) (50 mg) in MeOH (5·5 ml) and water (5 ml) was added to a MeOH (1 ml) solution of ethyl α-mercaptopropionate (75 μl) and the pH adjusted to 8–9 by 1N NaOH aq under N_2. After stirring for 2·5 hr at room temp., the mixture was acidified with dil. HCl and the ppt collected. Crystallization from a mixture of DMSO, MeOH and water gave fine yellow needles, m.p. 276–284° (dec) (40 mg). (Found: C, 49·36, 49·61; H, 2·91, 3·00; N, 10·15, 9·91. $C_{11}H_8N_2O_2S_2$ requires: C, 49·98; H, 3·05; N, 10·60%). IR (KBr) 1600, 1570; UV (MeOH) 378 (18,900); (MeOH–HCl) 378 (18,900); (MeOH–KOH) 436 (18,900); NMR (DMSO-d_6) 2·30 (3H, s), 7·05 (1H, d,d, J = 2·5, 9·0), 7·48 (1H, d, J = 2·5), 7·88 (1H, d, J = 9·0), 10·33 (2H, br; disappeared by addition of D_2O); *m/e* 264 (M^+). The product (IV) (28 mg) could also be obtained from X (49 mg) under the conditions used for the preparation of I.

2-(6'-*Hydroxybenzothiazol-2'-yl*)-5-*hydroxythiazolin*-4-*one* (probably a mixture of VIIa and VIIb). A solution of the nitrile (X) (216 mg) in MeOH (12 ml) and water (4 ml) adjusted to pH 8 with 1N NaOH aq was added to a solution of ethyl thioglycolate (300 μl) in MeOH (2 ml). After being stirred for 3 hr at room temp., the mixture was acidified with cold dil. HCl and the ppt collected (200 mg). Crystallization from MeOH aq followed by Avicel column chromatography (MeOH–H_2O, 7:3) (Avicel 120 g) gave a yellow crystalline powder, m.p. 192–193° (dec) (60 mg). IR: Fig 3; UV: Fig 4; NMR: Fig 5; MS: Fig 2. (Found: C, 45·12; H, 2·31; N, 10·52. $C_{10}H_6N_2O_3S_2$ requires: C, 45·10; H, 2·27; N, 10·52%).

(b) From I. To a solution of I (20 mg) in MeOH (10 ml) and water (2 ml) was added 1N NaOH (2 drops) and ethyl thioglycolate (20 μl) under N_2. After being stirred for 45 min. at room temp., the mixture was acidified with dil. HCl, and the ppt (15 mg) collected and crystallized from MeOH aq twice to give reddish-yellow crystalline solid, m.p. 193–195° (dec), whose IR spectrum was identical with that of VII obtained by procedure (a).

Diacetate of VII (VIII). VII (33 mg) was treated with Ac_2O (1 ml) and pyridine (1 ml) at room temp. overnight. Addition of water gave a yellow-brown ppt, which crystallized from MeOH to give VIII as a dark yellow crystalline solid m.p. 193–195° (dec) (11 mg). (Found: C, 48·24; H, 2·78; N, 7·82. $C_{14}H_{10}N_2O_5S_2$ requires: C, 47·99; H, 2·88; N, 7·99%). IR (KBr) 1770, 1725, 1605: Fig 6; NMR ($CDCl_3$): Fig 7.

2-(6'-*Hydroxybenzothiazol-2'-yl*)-4-*hydroxythiazole* (Oxyluciferin) (I). All processes were done under N_2 (or argon) and N_2 bubbled through the solvents before use.

A solution of ethyl thioglycolate (150 μl) in MeOH (4 ml) containing 300 μl of 1N NaOH aq was cooled in an ice bath and to this solution was added a cold solution of X (100 mg) in MeOH aq (60%, 22 ml). After being stirred for 2 min. under cooling, the mixture was acidified with dil. HCl and the ppt collected by filtration, washed with water and dried to give orange-yellow crystalline powder, m.p. 169–171° (dec) (85 mg). Attempted crystallization gave rather impure materials. (Found: C, 46·25, 46·52; H, 2·63, 2·63; N, 10·51, 10·46. $C_{10}H_6N_2O_2S_2 \cdot 1/2\ H_2O$ requires: C, 46·32; H, 2·72; N, 10·80%). IR (KBr): Fig 10; UV: Fig 12; NMR (DMSO-d_6): Fig 9; MS: *m/e* 250 (M^+): Fig 8; fluorescence spectrum: 564 (FWHM 2300 cm^{-1}) (in DMSO containing guanidine carbonate, in vac.): Fig 11.

Transformation of L-*luciferin to* IX. A suspension of L-luciferin (305 mg) in water (10 ml) sealed in glass tubing under reduced pressure was heated at 110° for 2 hr and the solution extracted with 10% $NaHCO_3$ aq.

The extracts were filtered and the filtrates acidified with dil. HCl to give a ppt which was washed with water and dried, as a yellow-brown crystalline powder, m.p. 157–157·5° (dec). (Found: C, 43·96; H, 3·27; N, 9·38. $C_{11}H_{10}N_2O_4S_2$ requires: C, 44·29; H, 3·38; N, 9·39%). IR (KBr) 3360, 1725, 1655, 1605, 1530; UV (MeOH) 262 (6200), 319·5 (1000); (MeOH–HCl) 262 (6200), 321 (9800); (MeOH–KOH) 282 (5250), 368 (10,600); NMR (DMSO-d_6) 3·05 (2H, br.d, J = 6·0), 4·55 (1H, m), 7·08 (1H, d.d, J = 2·5, 9·0), 7·47 (1H, d, J = 2·5), 7·95 (1H, d, J = 9·0), 8·88 (1H, d, J = 9·0; disappeared with addition of D_2O); MS: m/e 298 (M^+); Ninhydrin test: negative; α_D = 0·0 (c, 10·5 mg/4 ml MeOH).

Transformation of D,L-*luciferin to* IX. A suspension of D,L-luciferin (200 mg) in water (10 ml) was treated as above to give 133 mg of yellow-brown crystalline powder, m.p. 153·5–156° (dec). IR (KBr) 3300, 1720, 1645, 1605, 1520; NMR (DMSO-d_6) and R_f value of TLC were consistent with those of IX from L-luciferin. α_D = 0·0 (c, 11·5 mg/4 ml MeOH).

Transformation of oxyluciferin (I) *to luciferin and* IX. To a mixture of I (10 mg), water (1 ml) and 1N NaOH aq (90 μl) was added a mixture of L-Cys. HCl. H_2O (75 mg) and water (1 ml) adjusted to pH 7 with 1N NaOH aq, and then, the solution sealed under reduced pressure in glass tubing. After heating at 110° for 3 hr, the mixture was MeOH extracted and the extracts subjected to Avicel-Supercel TLC (MeOH–H_2O, 1:1). UV spectra of R_f 0·70 (YG) and R_f 0·76 (G) were superimposable to that of luciferin and IX, respectively. R_f 0·70 (YG): UV (MeOH) 327, 269; (MeOH–HCl) 330, 268; (MeOH–KOH) 384, 285; R_f 0·76 (G): UV (MeOH) 318, 262·5; (MeOH–HCl) 321, 262; (MeOH–KOH) 369, 282.

Formation of luciferin and IX *from the nitrile* (X) *and Cys.* To a solution of the nitrile (X) (12·5 mg) in MeOH (1·5 ml) was added a solution of L-Cys. HCl. H_2O (9 mg) in water (1 ml) adjusted to pH 8 with 1N NaOH aq. After stirring overnight at room temp., the mixture was separated by Avicel TLC (MeOH–H_2O, 1:1). Spots of R_f 0·73 (YG) and R_f 0·78 (G) were extracted with MeOH and their UV spectra measured, and were superimposable to that of luciferin and IX, respectively.

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