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STUDIES ON FIREFLY BIOLUMINESCENCE—II

IDENTIFICATION OF OXYLUCIFERIN AS A PRODUCT IN THE BIOLUMINESCENCE OF FIREFLY LANTERNS AND IN THE CHEMILUMINESCENCE OF FIREFLY LUCIFERIN*

N. SUZUKI† and T. GOTO

Faculty of Agriculture, Nagoya University, Chikusa, Nagoya, Japan

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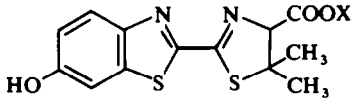
Abstract—Firefly oxyluciferin (II), 2-(6'-hydroxybenzothiazol-2'-yl)-4-hydroxythiazole, was identified as a product of firefly chemi- and *in vivo* bioluminescence.

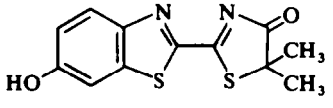
IT HAS BEEN suggested that the emitter in firefly bioluminescence and also in chemiluminescence of firefly luciferin (I) in DMSO would have structure II from consideration of the proposed reaction mechanism analogous to those of other chemiluminescent substances such as lophine and the acridinium carboxylic acids,[1] which involve a dioxetane intermediate,[2] and from an analogy with the product of chemiluminescence reaction of 5,5-dimethylluciferin derivatives. McCapra *et al.*[4]

Firefly Oxyluciferin II[3]

and Hopkins *et al.*[1,5] observed that when treated with strong base in DMSO, 5,5-dimethylluciferin derivatives (III, X = AMP or Ph) produced red luminescence and afforded thiazolinone IV, whose, fluorescence spectrum is identical with the chemiluminescence spectrum of III and with the fluorescence spectrum of the spent chemiluminescent reaction mixture.



III



IV

However, attempted isolation by White *et al.*[5,6] of the expected product, oxyluciferin (II), from the chemiluminescence reaction mixture and in the *in-vitro* bioluminescence mixture of firefly luciferin (I) has not been successful; they have

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† Present address: Faculty of Engineering, Mie University, Tsu, Japan.

recognized, instead, on the chromatograms of Sephadex LH 20 or silica gel three distinct spots designated as A, B and C, along with starting material (I) and dehydroluciferin (V), but no spot corresponding to II. Dehydroluciferin (V) could be produced by non-enzymatic air oxidation of luciferin.[7] They assumed that oxyluciferin (II) was first formed as a direct product of the chemi- and bioluminescence reactions but it was too unstable to be isolated and was rapidly converted into the three compounds. Although the mechanism of firefly bioluminescence is believed to be similar to that of firefly chemiluminescence,[1,4,5] recently, DeLuca *et al.*[8] reported that analysis of the O^{18} content in CO_2 formed during the *in vitro* bioluminescence of firefly luciferin conducted in O_2^{18} and H_2O^{18} gave results which contradict the mechanism involving dioxetane intermediate VI (Fig. 1).

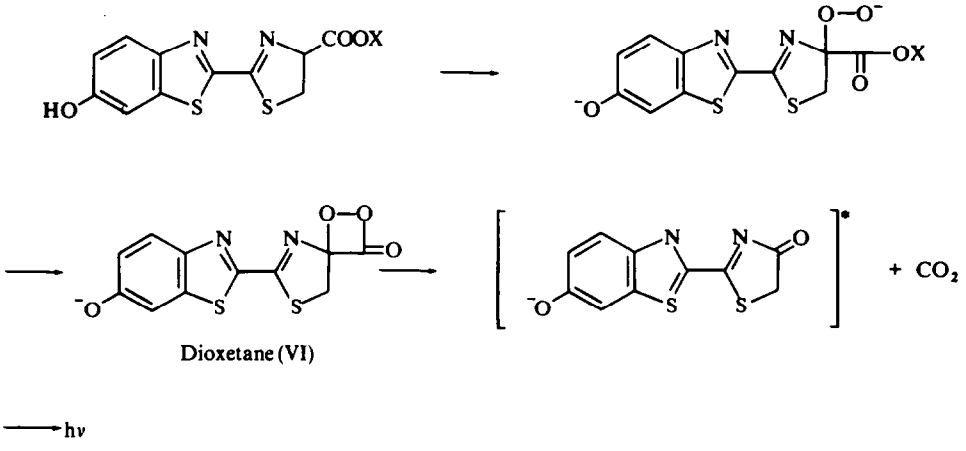


FIG. 1. The proposed mechanism of firefly chemiluminescence

To clarify the mechanism it is highly desirable to isolate and identify the product, oxyluciferin (II), other than CO_2 from the reaction mixture of chemi- and bioluminescence.

In our previous paper[3] the synthesis of a substance having structure II and the coincidence of the fluorescence spectrum of II and chemiluminescence spectrum of firefly luciferin (I) were reported. In this paper we report the isolation and identification of oxyluciferin (II) as a product of *in-vivo* firefly bioluminescence and of firefly chemiluminescence.

Isolation of oxyluciferin (II) *from a spent solution of chemiluminescence of luciferin* (I)

A solution of t-BuOK in t-BuOH was added to a solution of L-luciferin (optical antipode of natural luciferin) in DMSO, when light emission was observed, and the mixture evaporated *in vacuo*. TLC of the residue gave a blue fluorescent spot (R_f 0·10) identical in R_f value and color of fluorescence with those of synthetic II (Table 1). After elution with MeOH, the spot showed a UV spectrum identical with that of II (Fig. 2).

Further characterization was carried out as follows: the product of the above

TABLE 1. TLC OF PRODUCTS IN FIREFLY CHEMILUMINESCENCE

Solvent	II	I	V	Unknown
MeOH-H_2O 1:1	0·14[c] B[a] + +[b]	0·75 Y + + +	0·66 B +	0·29 B + 0·36 B + 0·43 B + 0·60 ±
95% EtOH-1N NH_4OAc 7:3	0·55[c] YG + +	0·59 YG + +	0·23 YG ±	0·84 B +
n-BuOH-HOAc-H_2O 4:1:4	0·73[c] B +	0·80 Y ±	0·58 Y ±	0·87 B ±

[a] Color of fluorescence; B: blue, Y: yellow, YG: yellowish green, G: green

[b] Avicel TLC 0·25 mm; detected by fluorescent lamp (366 nm). Intensities of fluorescence of oxyluciferin (II), luciferin (I) and dehydroluciferin (V) were similar in same concentrations. + + +, + +, + and ± show the intensities of fluorescence; + + +, very strong; + +, strong; +, present; ±, almost absent.

[c] This spot was not detected from the mixture without addition of t-BuOK.

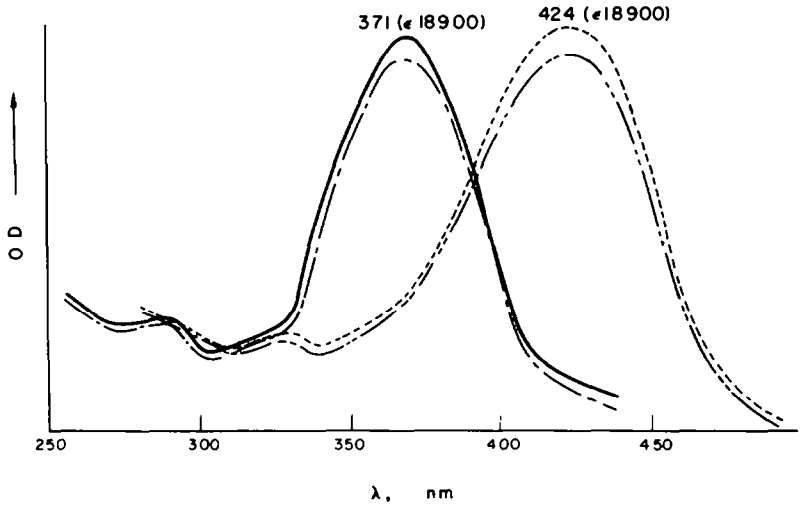


FIG. 2. UV spec. of oxyluciferin (II): Syn. ——— (MeOH) and ----- (MeOH-KOH); from chemiluminescence —·— (MeOH) and —··— (MeOH-KOH)

chemiluminescence reaction was immediately acetylated and then subjected to TLC separation on silica gel or Avicel. The product obtained showed UV (Fig. 3) and mass spectra (Fig. 4) identical with those of oxyluciferin diacetate (VII).

Incidentally, without addition of t-BuOK light was not produced and after the same treatment as above, II and VII were not detected.

Evidence for the formation of oxyluciferin (II) *during* in-vivo *bioluminescence of firefly*

The fireflies stored at −20° were allowed to stand at room temp., when they produced *in-vivo* bioluminescence. After emission ceased their lanterns were extracted with MeOH. TLC of the MeOH extracts on Avicel gave spots as shown in Table 2.

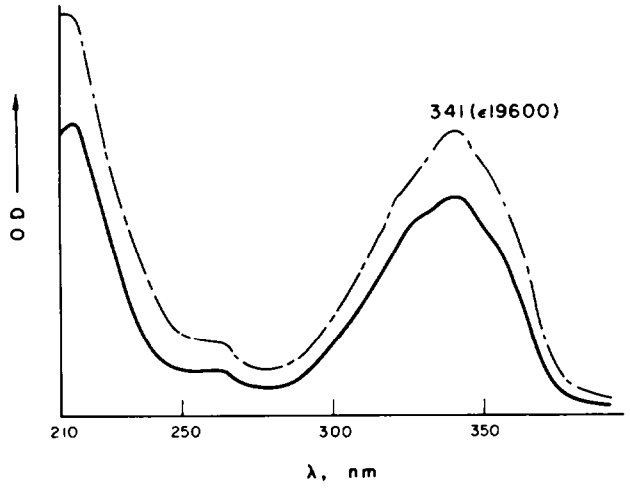


FIG. 3. UV spec. of oxyluciferin diacetate (VII) (MeOH): Syn. ———; from chemiluminescence — · —

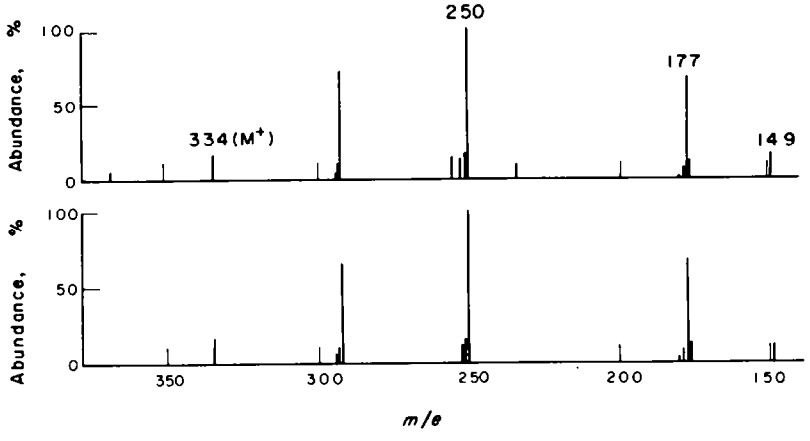


FIG. 4. MS of oxyluciferin (VII): from chemiluminescence (the upper) and syn. (the lower)

For comparison, the same extraction was also made without allowing bioluminescence. Identification was made by comparisons in R_f values and color of fluorescence on Avicel TLC plates with the authentic specimen (solv. systems used: MeOH-H_2O, 1:1; 95% EtOH-1N NH_4OAc, 7:3; n-BuOH-HOAc-H_2O, 4:1:4) (Table 2). Table 2 indicates the formation of oxyluciferin (II) during the *in-vivo* bioluminescence.

Attempted isolation of II directly from firefly lanterns was unsuccessful since repeated TLC of the oxyluciferin fractions led to the decomposition of II and large quantities of impurities disturbed chromatograms.

TABLE 2. TLC OF PRODUCTS IN FIREFLY BIOLUMINESCENCE

Solvent	II	I	V	XI	Unknown
MeOH-H_2O 1:1	0·12[c] B[a] + +[b]	0·80 YG +	0·60 B ±	0·78 V + + +	0·26 B + 0·42 B +
95% EtOH-1N NH_4OAc 7:3	0·86[c] YG +	0·64 YG + +	0·23 YG +	0·35 V + +	0·96 ±
n-BuOH-HOAc-H_2O 4:1:4	0·75[c] B +	0·81 Y + +	0·58 Y ±	0·16 Y + +	0·21 Y + 0·38 B ± 0·45 B ±

[a] Color of fluorescence; B: blue, Y: yellow, YG: yellowish green, G: green, V: violet.
[b] Avicel TLC 0·25 mm; detected by fluorescent lamp (366 nm). Intensity: see footnote (*b*) in Table 1.
[c] This spot was not detected in fireflies before bioluminescence.

Hence, isolation was carried out after acetylation of oxyluciferin (II). Repeated chromatographic separation (Table 3) of the acetylated products afforded the oxyluciferin diacetate (VII), which showed a UV spectrum identical with that of an authentic sample (Fig. 5).

TABLE 3. TLC OF PRODUCTS IN FIREFLY BIOLUMINESCENCE AFTER ACETYLATION

Avicel TLC (Avicel-Supercel 30:10) 1 mm

Solvent	II	Unknown
MeOH-H_2O 4:5	0·53[c] V[a] + + +[b]	0·00 + 0·49 +
95% EtOH-1N NH_4OAc 9:1	0·88[c] V + + +	0·84 ± 0·00 +
Silica gel TLC (Silica gel PF_{245}-gibshaltig) 1 mm		
Ether-CH_2Cl_2 10:1	0·84[c] V[a] + + +[b]	0·00 + 0·91 ±

[a] Color of fluorescence; V: violet.
[b] Avicel TLC (Avicel-Supercel 30:10) 1 mm; detected by fluorescent lamp (366 nm). Intensity: see footnote (*b*) in Table 1.
[c] This spot was not detected in the fireflies before bioluminescence.

The reason why dead fireflies do not contain oxyluciferin though they had bioluminesced whilst alive, could be because oxyluciferin was degraded during storage or that it did not accumulate in the bodies by metabolism. It might be used for re-synthesis of luciferin as suggested in the preceding paper.[3]

Oxyluciferin diacetate (VII) was synthesized as follows: 2-cyano-6-hydroxybenzothiazole,[7,9] when acetylated with Ac_2O and pyridine, gave 2-cyano-6-acetoxybenzothiazole (VIII), which was condensed with ethyl thioglycolate at pH 8 giving oxyluciferin monoacetate (IX). Its physical properties [elemental anal., IR (KBr) 1760 cm^{-1} (AcO), NMR (DMSO-d_6) 2·29 (3H, s) (AcO), 6·60 (1H, s) (olefinic H),

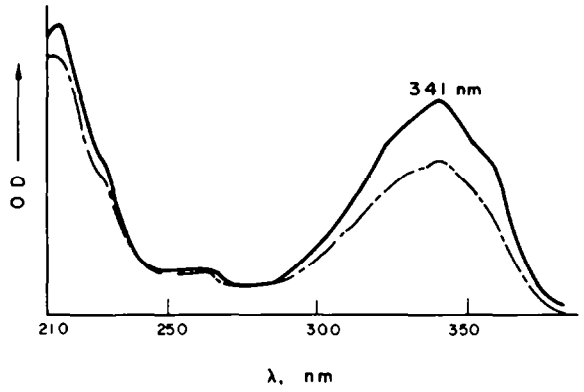
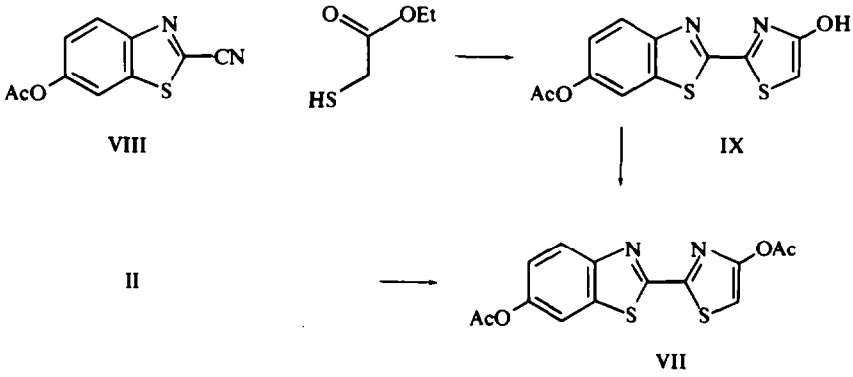


FIG. 5. UV spec. of oxyluciferin diacetate (VII) (MeOH): Syn. ——— and in-vivo bioluminescence —·—

11·1 (1H, br. s; disappeared with D_2O addition) (enolic OH)] shows that it must have structure IX. Acetylation of IX gave VII which was also obtained by acetylation of II: thus their structures are confirmed.



Hydrolysis of VII or IX, however, did not give II but resulted in a compound which seemed to be dioxyluciferin (X)[3] from its IR spectrum.

EXPERIMENTAL

All m.ps. were measured in sealed capillary tubes and are uncorrected.

The spectra were recorded on the following instruments: IR spectra Jasco IR-E; UV spectra: Hitachi EPS-3T; NMR spectra: JEOL JNM-4H-100; and Mass spectra: Hitachi RMU-6D spectrometer. Chem. shifts (δ) in ppm from int. TMS, coupling consts. (J) in Hz (accuracy $\pm$0·3 Hz).

Avicel TLC. Avicel (E. Merck AG.) (microcrystalline cellulose) (30 g) and Hyfro-supercel (10 g) were mixed with water (140 ml), spread with an applicator (thickness 1·0 mm) on glass plates (20 × 20 cm, 3 plates) and dried. Quantities of samples applied were 20–30 mg/plate.

Silica gel TLC. Silica gel PF_{254}-gibshaltig (E. Merck AG.) (70 g) and water (135 ml) were shaken for 2 min. and then spread on glass plates (20 × 20 cm, 2 plates) with an applicator (thickness 2·2 mm) and dried at 110° for 1 hr and stored in a desiccator.

L-**Luciferin** (I),[7] **oxyluciferin** (II),[3] **dioxyluciferin** (X)[3] and **dehydroluciferin** (V)[7] were synthesized according to the methods described in the literature.

Luciopterin (XI) was supplied by Kishi *et al.*[10]

2-Cyano-6-acetoxybenzothiazole (VIII). 2-Cyano-6-hydroxybenzothiazole[7,9] (605 mg) was treated with pyridine (6 ml) and Ac_2O (6 ml) and the mixture allowed to stand overnight at room temp. After evaporation *in vacuo*, the residue was crystallized from MeOH aq to give yellow needles, m.p. 125–127° (570 mg). (Found: C, 55·14; H, 2·86; N, 12·38. $C_{10}H_6N_2O_2S$ requires: C, 55·04; H, 2·77; N, 12·84%). IR(KBr) 2240, 1760; NMR (acetone-d_6) 2·29 (3H, s), 7·41 (1H, d.d, J = 2·5, and 9·0), 8·02 (1H, d, J = 2·5), 8·19 (1H, d, J = 9·0); UV (MeOH) 292 (13300), 348 (8050).

2-(6'-Acetoxybenzothiazol-2'-yl)-4-hydroxythiazole (IX). A solution of VII (400 mg) and ethyl thioglycolate (0·8 ml) in MeOH (70 ml) was adjusted to pH 8 by addition of 1N NaOH aq and stirred at room temp. for 1 hr. The solv. was removed and the residue crystallized from MeOH aq as yellow crystalline powder, m.p. 195–198° (351 mg). (Found: C, 49·49; H 2·52; N, 9·88. $C_{12}H_8N_2O_3S_2$ requires: C 49·30; H, 2·76; N, 9·58%) IR (KBr) 1760, 1605, 1580, 1560; UV (MeOH) 361 (20400), MeOH-HCl) 361 (20400), (MeOH-KOH) 424 (23800); NMR (DMSO-d_6) 2·29 (1H, s), 6·60 (1H, s), 7·34 (1H, d.d, J = 2·5, 9·0), 7·97 (1H, d, J = 2·5), 8·08 (1H, d, 9·0), 11·1 (1H, br. s; disappeared by D_2O addition).

VIII was hydrolyzed with NaOH aq to give a crude product whose IR spectrum was almost superimposable with that of dioxyluciferin (X).

2-(6'-Acetoxybenzothiazol-2'-yl)-4-acetoxythiazole (VII). (a) From VIII. The monoacetate (VIII) (10 mg) was acetylated with pyridine (1 ml) and Ac_2O (1 ml) under N_2. Crystallization from MeOH gave yellow leaflets, m.p. 178–181° (10·5 mg). (Found: C, 50·30; H, 2·78; N, 8·10. $C_{14}H_{10}N_2O_4S_2$ requires: C, 50·29; H, 3·01; N, 8·38%). IR (KBr) 1760; UV (MeOH) 341 (19600) (Fig. 5); NMR ($CDCl_3$) 2·36 (3H, s), 2·39 (3H, s) 7·27 (1H, d.d, J = 2·5, 9·0), 7·35 (1H, s), 7·73 (1H, d. J = 2·5), 8·08 (1H, d, J = 9·0); MS: *m/e* 334 (M^+).

(b) From II. Oxyluciferin (II) (8·5 mg) was acetylated as above to give pale yellow leaflets (10·1 mg), whose IR spectrum was identical with that from VIII.

Hydrolysis of VII with NaOH aq gave the same product as that obtained from VIII.

Isolation of II *from a mixture of firefly chemiluminescence.* Into a DMSO (5 ml; saturated with O_2) solution of L-luciferin (I) (56 mg) was added a mixture of DMSO (95 ml) and t-BuOH (0·4 ml) containing 0·5 M t-BuOK. After stirring for 40 min at room temp, excess dry ice was added to neutralize remaining base. Solvents were evaporated *in vacuo* (10^{-5} mm Hg) and the residue extracted with MeOH. The extracts, after evaporation to dryness, were chromatographed on Avicel (Table 1). The spot R_f 0·10 on TLC (MeOH-H_2O, 1:1) was eluted with MeOH and its UV spectrum measured (Fig. 2).

The above experiment was repeated without addition of t-BuOK (Table 1).

Isolation of VII *from a mixture of firefly chemiluminescence.* Into the above mentioned DMSO mixture, after chemiluminescence, were added Ac_2O (2 ml) and pyridine (1 ml) at 0° and the mixture stirred for 4 hr at room temp. After evaporation of solvents *in vacuo* (10^{-5} mm Hg) at room temp, the residue was ether extracted and the extracts chromatographed on silica gel (10 × 20 cm, 2·2 mm, 5 plates, ether–CH_2Cl_2, 10:1). The fraction R_f 0·84 was eluted with ether and rechromatographed on Avicel (20 × 20 cm, 1·0 mm, 7 plates, MeOH-H_2O, 4:5) and the fraction R_f 0·53 was eluted with ether. After evaporation of solvents, the UV and mass spectra of the residue were measured (Fig. 3 and 4, respectively).

When the above treatment was repeated on the DMSO solution of L-luciferin (I) without addition of t-BuOK, the fluorescent spot corresponding to VII was not detected.

Confirmation of production of II *during firefly* in vivo *bioluminescence.* Japanese fireflies (Genji-hotaru; *Luciola cruciata*, collected in Gifu pref. in Japan, 1969), killed by dipping in dry ice and stored in a deep-freeze (−20°), were used in the following experiments.

Fireflies (7 individuals of female) were allowed to stand for 15 min at room temp (*ca.* 22°, light produced) and their lanterns cut off. After grinding them in MeOH (cooled by direct addition of dry ice), the slurry was filtered and the filtrate evaporated *in vacuo* (10^{-5} mm Hg). The residue was chromatographed on Avicel TLC plates. (Table 2).

The above experiments were repeated using fireflies without allowing to stand at room temp. (TLC data, Table 2).

Isolation of VII *as a product of firefly* in vivo *bioluminescence.* After fireflies (100 individuals) were allowed to stand for 15 min at room temp, their lanterns were ground in Ac_2O (10 ml) and pyridine (2 ml) under external cooling with dry ice and then allowed to stand for 1·5 hr at room temp. After solvents were evaporated *in vacuo*, the residue was ether extracted and the extracts chromatographed on Avicel (10 × 20 cm, 1·0 mm, 7 plates, MeOH-H_2O, 1:1). The fraction R_f 0·70 was eluted with ether and rechromatographed on silica gel (20 × 20 cm, 2·2 mm, 3 plates, ether-CH_2Cl_2, 10:1). The results are shown in Table 3.

After repeated chromatography (3 times) on silica gel the R_f 0·84 spot was eluted with ether and its UV spectrum measured (Fig. 5).

The above experiment was repeated using fireflies not allowed to stand at room temp, (TLC data, Table 3).

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