Chemi- and Bioluminescence of Firefly Luciferin

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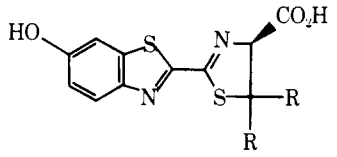
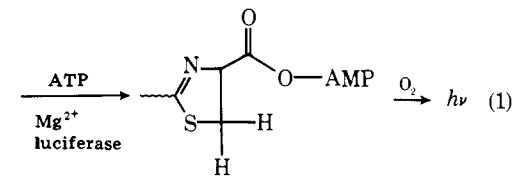
Abstract: Chemiluminescence of the ethoxyvinyl ester of 5,5-dimethylluciferin (**3b**) with base and $^{18}O_2$ gave carbon dioxide in 39% yield enriched with 57–83% of ^{18}O. The corresponding experiment with luciferin (**3a**) gave carbon dioxide in 20% yield with 76% ^{18}O incorporation. The ^{18}O incorporation substantiates a dioxetanone mechanism for the light-producing pathway. The products from 5,5-dimethylluciferin ester **3b** were 5,5-dimethyloxyluciferin (**5b**, 30%), dimethylluciferin (**1b**, 12%), and an isopropylidene rearrangement compound, **6** (44%). For firefly luciferin ester (**3a**), the products were oxyluciferin (**5a**, 30%) and dehydroluciferin (**7**, 73%). Excited-state yields of the oxyluciferins produced ranged from 33 to 53%. In contrast to the above results with "active" esters, the methyl ester of luciferin (**8**) on oxidation did not produce light and the major product formed was 4-hydroxyluciferin methyl ester (**10**). The formation of dehydroluciferin is discussed.

Bioluminescence in the firefly is a complex phenomenon based on a simple set of chemical reactions of luciferin (**1a**),



1a, R = H
b, R = CH_3



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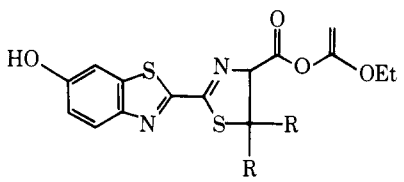
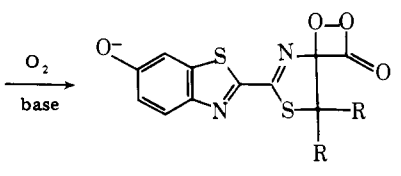
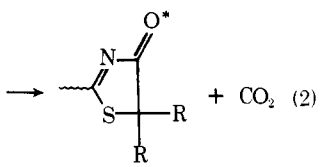
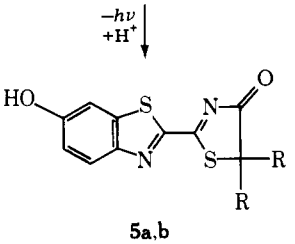


3a, R = H
b, R = CH_3



4a,b





5a,b

ATP, oxygen, and magnesium ion catalyzed by the enzyme luciferase. Two steps are involved in this chemical production of excited states: (1) mixed anhydride formation to yield luciferyl AMP and pyrophosphate[1] and (2) oxidation of the luciferyl adenylate[2] (eq 1). Luciferyl AMP is also chemiluminescent on reaction with a base and oxygen, and other simpler "esters" are also active.[3] Work with the latter compounds led to the reaction mechanism for the chemiluminescence of luciferin given in eq 2,[4,5] which was also proposed to hold for the bioluminescence;[4] preliminary results of our ^{18}O study of the chemiluminescence were consistent with the mechanism.[6]

The chemically produced excited states were proposed to arise from a dioxetanone (**4**). This view was based on earlier studies of a common type of chemiluminescence in which oxygen is required at some stage and in which dioxetanes are critical precursors of excited states.[7]

In the meantime, five contributions by De Luca et al. have appeared purporting to rule out the dioxetanone mechanism for both chemi- and bioluminescence.[8] Oxygen isotopes were used and the results appeared to indicate that none of the oxygen atoms of the product carbon dioxide stemmed from the oxygen consumed. These conclusions have been widely cited.[9] Several defects in these studies have been pointed out which vitiate the mechanistic claims.[4b,6] The chief difficulties are (1) the chain of evidence was not completed in the work; that is, quantum yields and chemical yields, e.g., were assumed to be the same as values measured under nonidentical conditions in other laboratories, and (2) no proof was provided that the authors could recover the microliter quantities of carbon dioxide which had been produced from the reaction media. In the chemiluminescence experiments, at least, it appears that adventitious carbon dioxide was analyzed throughout, since the claim was made that carbon dioxide produced in the reaction of luciferyl esters with potassium *tert*-butoxide was pumped directly out of the reaction mixture.[10] This is an impossible act in view of the high rate of reaction of *tert*-butoxide ion with carbon dioxide in solution and the great stability of the alkali metal salts of monoalkyl carbonates. In our own studies no detectable carbon dioxide was released from such basic solutions.

In contrast to the DeLuca conclusions, Johnson and Shimomura have shown in a study[11] of firefly bioluminescence that the carbon dioxide formed contains one atom of oxygen derived from the oxygen consumed. Recently, DeLuca et al.[12] have reinvestigated their earlier work and now conclude that the carbon dioxide formed in firefly bioluminescence does indeed contain an atom of oxygen derived from the oxygen. Thus, the general dioxetane-dioxetanone mechanism (eq 1 and 2) for both the bio- and chemiluminescence of firefly luciferin appears to be secure.

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Table I. Products from the Chemiluminescence of Luciferin Ethoxyvinyl Ester **3a**[a,b]

run	solvent	base	base/ ester	O_2, mm	**5a**, %	**7**, %	CO_2, %
1	Me_2SO	PhOK	72	228[c]	34	46	
2	Me_2SO	*t*-BuOK	3	760	27	54	
3	Me_2SO	*t*-BuOK	18	760	30	73	
4 (5)	THF	*t*-BuOK	18	760	49 (42)	20 (22)	
6 (7)[d]	Me_2SO	PhOK	67	209	16 (17)	51 (55)	22 (17)

[a] Concentration of ester was 5–8 × 10^{-4} M. [b] Values of duplicate runs in parentheses. [c] Air-saturated solution. [d] Vacuum-line techniques employed.

Table II. Dependence of Chemiluminescence Quantum Yields for Luciferin Ethoxyvinyl Ester (**3a**) on Oxygen Pressure

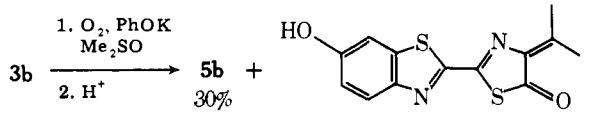
O_2, mm	Φ_{CL}(rel)[a]
760	1.0
374	1.0
217	1.0
115	0.70
65	0.35

[a] Quantum yield relative to that at 760 mm O_2.

In the present paper we give full details of our ^{18}O study of the 5,5-dimethyl derivative of luciferin,[13] and also an account of parallel studies of the parent luciferin.

Results

Products. Carboxylic acid derivatives of luciferin with good leaving groups produce a brilliant chemiluminescence on treatment with a base and oxygen. This reaction in the case of the ethoxyvinyl ester of 5,5-dimethylluciferin (**3b**) leads to 5,5-dimethyloxyluciferin (**5b**), a rearrangement product (**6**), 5,5-dimethylluciferin (**1b**), carbon dioxide (eq 3), and a small amount of an unknown substance.[6a,14]



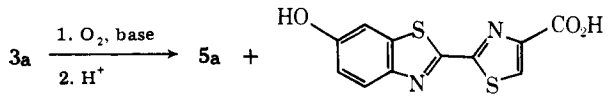
44%

6

+ **1b** + CO_2 + $h\nu$ (3)

12% 39%

The chemiluminescent reaction of the ethoxyvinyl ester of the parent luciferin (**3a**) with base and oxygen produced oxyluciferin (**5a**),[15] dehydroluciferin (**7**),[16] carbon dioxide, and an unidentified degradation product (eq 4 and Table I); interestingly, the product distribution appeared to be solvent dependent. The analogue of compound **6** was apparently not formed in the parent luciferin case. Firefly luciferin is stable under the reaction conditions and it is not the source of dehydroluciferin. Yields of carbon dioxide were obtained during the ^{18}O tracer study using vacuum line techniques and are summarized in Table I (runs 6 and 7).

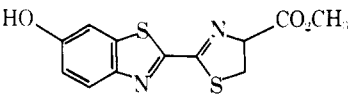


7

+ CO_2 + $h\nu$ (4)

In the absence of oxygen, the reaction of luciferin ester **3a** with base was nonchemiluminescent; neither oxyluciferin (**5a**) nor dehydroluciferin (**7**) was produced, the hydrolysis product, luciferin, being the only product detected.

The methyl ester of luciferin (**8**) was treated with potassium phenoxide and oxygen under similar conditions but no light



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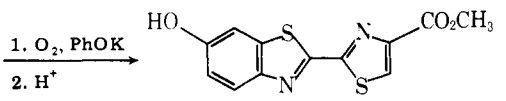

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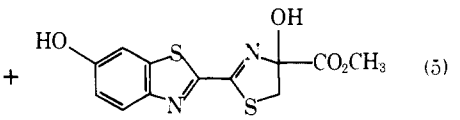


(5)

10

was produced; the products were the methyl ester of dehydroluciferin (**9**, 17% yield) and the methyl ester of 4-hydroxyluciferin (**10**, 48% yield) (eq 5). The major product (**10**) was characterized by its spectral data and transformation to dehydroluciferin methyl ester (**9**) by acid-catalyzed dehydration. Dehydroluciferin methyl ester (**9**) was not formed from hydroxy ester **10** under the reaction conditions employed for ester **8** oxidation, nor during workup and product isolation.

Chemiluminescence. The chemiluminescence maximum for the ethoxyvinyl ester of 5,5-dimethylluciferin (**3b**) was found to be 631 nm with potassium phenoxide, potassium *tert*-butoxide, or potassium imidazolate as the base in dimethyl sulfoxide solutions saturated with oxygen. The wavelength was found to be independent of base type or concentration.[4] The quantum efficiency, Φ_{CL}, with a tenfold excess of base was 0.09 for dilute solutions (10^{-5} M).[4,6a,14]

The chemiluminescence spectrum of the ethoxyvinyl ester of firefly luciferin (**3a**) was similar to that of other luciferin derivatives,[4] exhibiting a λ_{max} of 625 nm (red light) in dimethyl sulfoxide with 1.63 × 10^{-3} M potassium phenoxide as the base (12.5 equiv). In tetrahydrofuran, an orange emission at 595 nm was observed under these conditions.

For the ethoxyvinyl ester of firefly luciferin (**3a**), the Φ_{CL} was 0.09 at 1 × 10^{-5} M ester. The dependence of quantum yield on oxygen pressure was determined (Table II) in order to optimize conditions for the $^{18}O_2$ tracer study; for 5.71 × 10^{-4} M solutions of ester **3a**, decreased quantum yields were observed for oxygen pressures less than 150 nm.

Fluorescence Measurements. 5,5-Dimethyloxyluciferin (**5b**) exhibited a long-wavelength absorption at 580 nm in dimethyl sulfoxide containing an excess of potassium phenoxide (300 equiv), with fluorescence occurring at 633 nm. Similar results were reported for potassium imidazolate or triethylamine as the base.[4]

The absorption and fluorescence spectra of firefly oxyluciferin (**5a**) proved to be dependent on base concentration (Table III). With a large excess of potassium phenoxide in dimethyl sulfoxide, the absorption λ_{max} of **5a** was 475 nm, and excitation at this wavelength produced a single yellow-green fluorescence

Table III. Fluorescence of Firefly Oxyluciferin (**5a**), Dehydroluciferin (**7**), and Spent Reaction Mixtures

compd	base/compd ratio	absorption λ_{max}, nm	fluorescence λ_{ex}, nm	fluorescence λ_{em}, nm
5a[a]	300	475	475	570
5a[a]	1.4	497, 570 (sh)	497	570, 610 (sh)
			570	628
5a[a]	0.27	375, 497, 570 (sh)	497	570, 610 (sh)
			570	628
5a[a]	0.0	375	375	428
spent chemiluminescence reaction mixture[b]	2.0	472, 570 (sh)	472	555
			570	628
dehydroluciferin (**7**)	300	471	471	552

[a] 9.03×10^{-5} M in argon-saturated dimethyl sulfoxide. [b] From 2.09×10^{-4} mmol of ester **1b** and 2 equiv of potassium phenoxide in 3.5 mL of dimethyl sulfoxide, flushed with argon for the measurements.

Table IV. ^{18}O Incorporation in the Carbon Dioxide Produced from Luciferin Esters **3a,b**[a-c]

run	ester	base	% CO_2 labeled with ^{18}O[d]
8	**3b**	*t*-BuOK	83
9	**3b**	*t*-BuOK	67
10	**3b**	*t*-BuOK	57
11	**3a**	PhOK	76

[a] Vacuum-line techniques used. [b] Ester and excess base in dimethyl sulfoxide at 25 °C. [c] 100% phosphoric acid for acidification except for run 11, which used phenol. [d] Raw values corrected for the atom % ^{18}O in the starting oxygen, but not for exchange.

emission at 570 nm. At a base:**5a** ratio of 1.4:1 the absorption spectrum gave λ_{max} 497 nm with a less intense shoulder at 570 nm. Excitation into the short-wavelength band gave a fluorescence emission at 570 nm with a shoulder at 610 nm. Excitation into the weak longer wavelength shoulder gave only red emission (628 nm). With less than 1 equiv of base, an absorption at 375 nm was present (un-ionized oxyluciferin) along with the 497- and 570-nm bands; the fluorescence spectrum was similar to that obtained with 1.4 equiv of base.

A spent ester **3a** chemiluminescence reaction mixture also exhibited a 570-nm shoulder in the absorption spectrum; excitation into the shoulder gave red emission at 628 nm paralleling the oxyluciferin (**5a**) result (Table III). The major remaining bands were assigned to dehydroluciferin (Table III). An additional 2.6 equiv of base caused disappearance of the 570-nm absorption and 628-nm emission, whereas the 555-nm emission of **7** remained unchanged. Acidification of the spent reaction mixture with glacial acetic acid gave a spectrum identical with that of dehydroluciferin (**7**) (λ_{max} 350 nm).

Tracer Studies. Our product study[14] of 5,5-dimethylluciferin ester (**3b**) chemiluminescence showed that oxygen was necessary for 5,5-dimethyloxyluciferin (**5b**) formation and also for light emission; the same result was obtained for luciferin ester **3a** chemiluminescence.

In the tracer experiments, base was added to a solution of an ester of luciferin containing dissolved $^{18}O_2$. After light emission ceased, an acid was added to liberate the carbon dioxide from the carbonate salt which had been formed in the basic medium and the carbon dioxide was analyzed. In a regular apparatus sealed by rubber septa, the ^{18}O incorporation was low (1–16%) in the carbon dioxide produced following acidification with strong acids even when precautions were taken to avoid traces of water and atmospheric carbon dioxide. The highest incorporation (22%) was obtained when anhydrous phosphoric acid[17] was used. In the same run 60% of the total 5,5-dimethyloxyluciferin was labeled with ^{18}O. In a control run, labeled CO_2 was carried through one acidification cycle with the result that 62% of the ^{18}O was "washed out". Thus, 58% of the CO_2 from the luciferin would have been labeled prior to acidification, corresponding to the percent of the 5,5-dimethyloxyluciferin that was labeled.

In order to minimize the problem of exchange by adventitious water,[18] vacuum-line techniques were used in which all manipulations such as purification, transfer of reagents, mixing, and initiation of chemiluminescence were performed in a closed system. The yield of isotopic enrichment in the carbon dioxide increased dramatically under these conditions to 83% in one case, and it averaged 72% (runs 8–10, Table IV). Comparable enrichment in ^{18}O was found in the isolated 5,5-dimethyloxyluciferin (94% ^{18}O).

In one run, $H_2{}^{18}O$ was added to the reaction mixture and $^{16}O_2$ was used as the oxidant. Serious exchange occurred to give carbon dioxide containing 52% $C^{16}O^{18}O$ and 34% $C^{18}O^{18}O$, but some unlabeled CO_2 remained (14%).[18b]

Carbon dioxide obtained from the chemiluminescence of the ethoxyvinyl ester of the parent firefly luciferin (**3a**) had an isotopic enrichment of 76% ^{18}O (Table IV). In this case a large excess of phenol relative to potassium phenoxide was used to buffer the medium so that carbon dioxide could be collected without resorting to strong acid to decompose the intermediate potassium phenyl carbonate. Improvement over the 100% phosphoric acid method used in ester **3b** runs was not realized, however.

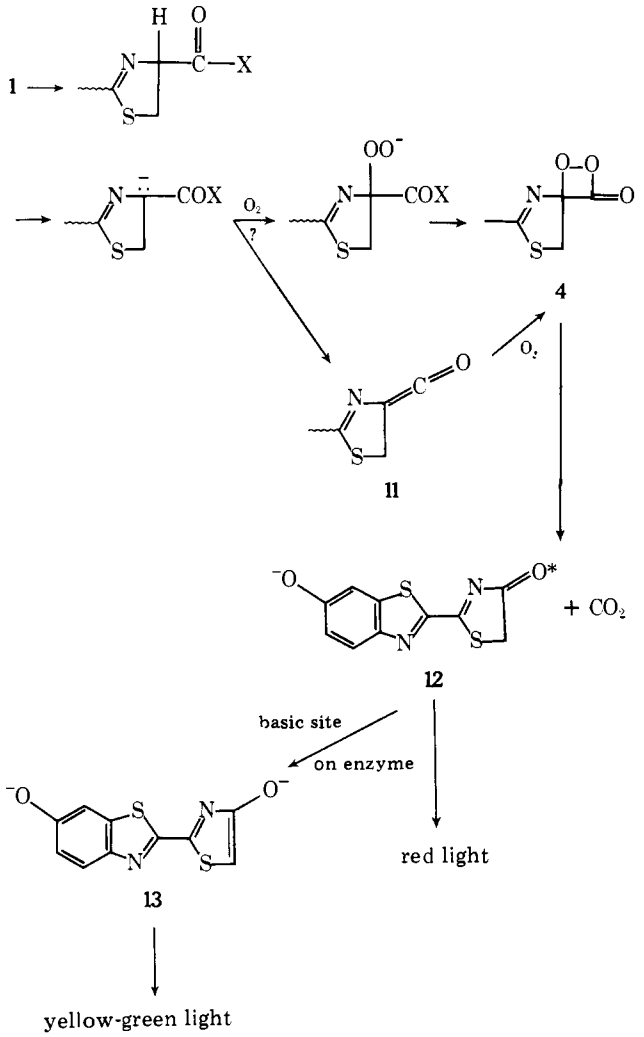
Discussion

The data will be discussed in terms of the general mechanism for firefly luminescence proposed earlier (Chart I).[4] The ethoxyvinyl esters of the luciferins (**3**) were used in this study since they could be readily prepared analytically pure; the adenylate analogue (**2**) has not been reported as an isolable, pure solid and the phenyl ester is difficult to prepare.[19]

The Light Emitter in Luciferin Chemi- and Bioluminescence. Yellow-green light (ca. 560 nm) is normally produced by fireflies, but under certain conditions (e.g., at higher temperatures) red light (ca. 630 nm) is emitted.[20a] The in vitro luciferase-catalyzed luciferin oxidation also yields yellow-green light normally, but red-light emission occurs at low pHs, at higher temperatures, and in the presence of heavy metals.[2] The chemiluminescence of the ethoxyvinyl ester of firefly luciferin **1a**, on the other hand, yields only red light under a variety of conditions.[21] Whether red or yellow-green light is emitted depends on whether ionization of **12** to **13** can compete with emission (Chart I). In the chemiluminescence of ethoxyvinyl ester **3a**, external base does not compete and only red emission is observed. In the luciferase-catalyzed reaction, a basic group on the enzyme does compete, and yellow-green emission is normally seen. At low pHs, red light is produced presumably because a basic group on the enzyme is being protonated. When that basic group is alkylated, it has been found that only red-light emission occurs irrespective of the pH of the medium.[22]

In the case of 5,5-dimethylluciferin, the anion of the oxy-

Chart I. Firefly Luciferin Luminescence



~X = AMP (**2**), OC_6H_5, $OC(=CH_2)OC_2H_5$ (**3**), etc.

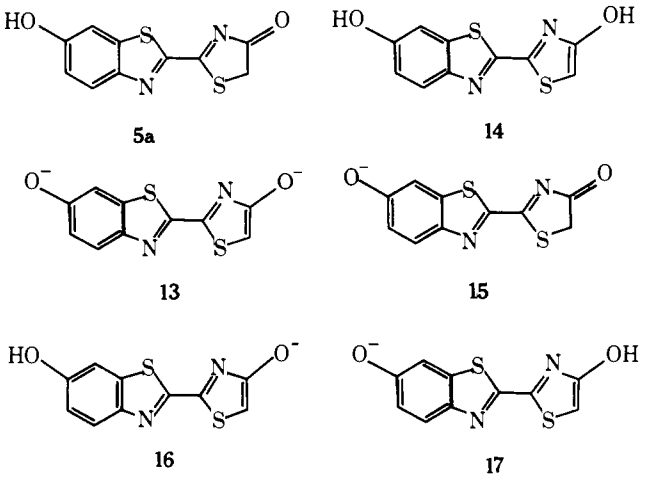
luciferin formed has no further tautomeric or proton-transfer possibilities and only red-light emission is possible. The chemiluminescence of the ethoxyvinyl ester of 5,5-dimethylluciferin (**3b**), the fluorescence of spent reaction mixtures, and the fluorescence of synthetic 5,5-dimethyloxyluciferin (**5b**) in basic solutions all give a maximum in the red region of the spectrum at 633 nm.

Native luciferin has complex light emission possibilities since the corresponding oxyluciferin (**5a**) is a dibasic acid capable of tautomerism and several species differing by one or two protons are possible (Chart II).

Oxyluciferin dianion **13** was proposed as the yellow-green light emitter in the enzyme-catalyzed reactions on the basis of fluorescence measurements on oxyluciferin (**5a**) prepared in situ,[4] and later verified with isolated material.[15a,e] In strongly basic solutions, synthetic oxyluciferin fluoresces in the yellow-green region of the spectrum at 570 nm. This wavelength is essentially the same as that observed in yellow-green bioluminescence.[4]

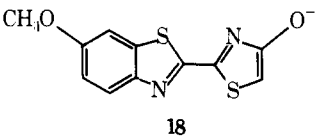
At low ratios of base to oxyluciferin (**5a**) (1.4/1, see Table III), broad absorption maxima are observed for oxyluciferin at 497 and 570 nm. Excitation at 570 nm leads to emission at 628 nm in the red region of the spectrum. The chemiluminescence of the ethoxyvinyl ester of luciferin (**3a**) shows a maximum emission at 625 nm, and one of the fluorescence emission bands of the spent chemiluminescence reaction mixture is at 628 nm (Table III). Since 5,5-dimethyloxyluciferin monoanion

Chart II. Forms of Oxyluciferin



fluoresces at 633 nm, the 625–628-nm emissions cited in the three cases above are assigned to keto monoanion **15** (Chart II).

When excitation of the low-base mixture is carried out at 497 nm, emission occurs at 570 and 610 nm (Table III); the 570-nm emission probably stems from **13** and the 610-nm emission (16 400 cm^{-1}, orange) from either species **16** or **17**.



Consistent with the latter assignment is the report that compound **18** fluoresces in the orange range of the spectrum (16 900 cm^{-1}).[4b] Enzymic control of the interconversion of the various species in Chart I could lead to light emission over a considerable range of wavelengths; the well-known observation that different species of fireflies emit light at appreciably different wavelengths[20b] may be a result of such control.

Yields of Excited States. The quantum yield of chemi- or bioluminescence can be dissected into three components each having a probability of occurring: (1) the fraction of reaction that produces the potential light emitter, Φ_r, (2) the fraction of the potential light emitter that is formed in its excited state, Φ_{ex}, and (3) the fraction of excited states that emit light, Φ_{fl} (quantum yield of fluorescence). The overall probability for the occurrence of luminescence, Φ_{CL}, is given by the equation

$$\Phi_{CL} = \Phi_r \Phi_{ex} \Phi_{fl} \quad (6)$$

In the luciferase-catalyzed bioluminescence of native firefly luciferin in vitro, the efficiency for light production has been reported to be 88 ± 12%. A quantum yield this large requires that each component (Φ_r, Φ_{ex}, Φ_{fl}) be nearly 100% efficient and thus the yield of chemically produced excited states in this system must be close to 100%.

In the chemiluminescence of the ethoxyvinyl ester of 5,5-dimethylluciferin (**3b**), 28% of dimethyloxyluciferin (**5b**) was isolated; 56% of the reaction led to the isopropylidene rearrangement product **6** and to 5,5-dimethylluciferin (**1b**) in dark reactions. The yield of oxyluciferin thus falls between 28 and 44% and Φ_r has a value between 0.28 and 0.44. Of course, the oxyluciferin could also be formed in a second, "dark", competing process, but, as there is no way of assessing and correcting for this possibility, an "operational" definition of Φ_r—as used above—seems reasonable. The quantum yield of fluorescence, Φ_{fl}, for 5,5-dimethyloxyluciferin (**5b**) in basic dimethyl sulfoxide is 0.62 (Experimental Section). Using the Φ_{CL} of 0.09 (Results section) and eq 6, Φ_{ex} is calculated to be

0.33 for $\Phi_r = 0.44$ and 0.53 for $\Phi_r = 0.28$. Thus, the oxidation pathway leading to oxyluciferin yields between 33 and 53% of singlet excited states of 5,5-dimethyloxyluciferin (**5b**).

The excited-state yield for the ethoxyvinyl ester of native luciferin **3a** could not be determined readily. The luminescence is red colored with potassium phenoxide as base, and presumably oxyluciferin species **15** (Chart II) is the light emitter. The fluorescence quantum yield of **5a** was not determined since it exists in equilibrium with several other species (**13–17**, see Chart II) and neither the equilibrium constants nor the light-emitting abilities of the various species are known.

^{18}O Incorporation and Mechanism of the Reaction. When the chemiluminescence of luciferin ester **3a** was carried out in a closed system using vacuum-line techniques, the directly measured ^{18}O incorporation in the carbon dioxide rose to 57–83% and the ^{18}O incorporation in lactam **5b** rose to 94%. For ester **3a**, the ^{18}O incorporation in the CO_2 was 76% (Table IV) in vacuum-line work. To eliminate oxygen exchange in the liberation of the carbon dioxide, alkylation of the potassium *tert*-butyl (or phenyl) carbonate with methyl iodide and mass-spectral analysis of the volatile carbonate ester were attempted, but abandoned because of the low yields obtained.

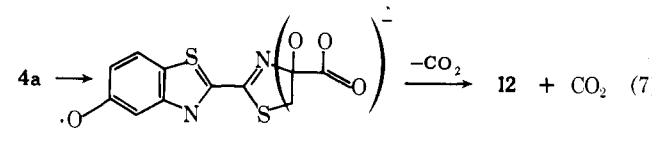
Focusing attention on the pathway leading to oxyluciferin, we have shown for the chemiluminescence of the ethoxyvinyl ester of dimethylluciferin (**3b**) that the total amount of carbon dioxide formed is 39% (eq 3 and Experimental Section). Correcting this value for the rearrangement compound (44%) and the amount of hydrolysis (12% of **1b**), the yield of carbon dioxide in the oxidative pathway is calculated to be 89%. Since the yield of excited states for the excitation path was determined to be 33–53%, and since 89 + 33 > 100%, carbon dioxide must be a product of the light-producing pathway. The similarity of the oxyluciferin (30%) and carbon dioxide (39%) yields is consistent with the proposed reaction (eq 1).

On the average, 70% of the carbon dioxide molecules formed in the vacuum line runs contained an atom of ^{18}O (Table IV). Since a minimum of 33% excited states was formed in the oxidative pathway, the data is at least consistent with the view that ^{18}O is incorporated in the carbon dioxide during the generation of excited states. In the run with the least washing out of the label, the ^{18}O incorporation was 83%; since 83 + 33 > 100%, an overlap occurs and excited states must have been formed with ^{18}O incorporation.

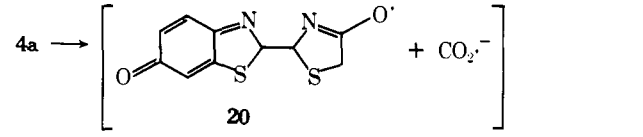
The products formed, their distribution, the spectral data, and the labeling data are all consistent with the view that dioxetanone intermediates are involved in the chemiluminescence of firefly luciferin (Chart I);[24] the bioluminescence of firefly luciferin also follows this pathway.[11,12]

As an alternative to the reaction pathway illustrated in the upper pathway of Chart I, an elimination of a type well known for acid chlorides might occur to give a ketene (**11**). The formation of dioxetanones from ketenes and oxygen has been reported.[25]

High yields of singlet states are obtained in the chemi- and bioluminescence of firefly luciferin. Yet simple dioxetanones and dioxetanes decompose to give principally triplet states. In an effort to explain the high singlet yields, Koo, Schmidt, and Schuster[26] (eq 7), and also McCapra[27] (eq 8), have proposed electron-transfer mechanisms for the generation of excited states from dioxetanones of luciferin;[28] reaction mechanisms were not specified. As a point of interest, the dioxetanone ring in **4** defines a plane perpendicular to the plane of the conjugated benzothiazole and thiazoline rings. To be analogous to the intermolecular electron transfer processes proposed for the chemiluminescence of dioxetanones plus fluorescent compounds,[26] the formation of **19** would require through-space electron transfer between the mutually perpendicular rings of **4**. Some analogy for this type of transfer exists in the work of



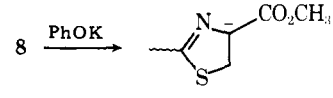
19



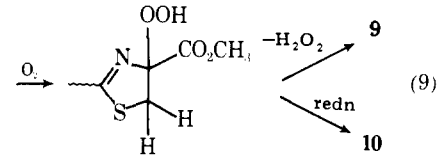
20

Simmons and Fukunaga[31] on electron transfer in spiro systems.

Dehydroluciferin (7). The title compound has been detected in fireflies, it can be isolated from that source,[32] and it has been prepared by the air oxidation of luciferin in aqueous base.[16] We now find that the methyl ester of dehydroluciferin, **9**, is formed in the air oxidation of the methyl ester of luciferin; no light is produced in these oxidations. The oxidation probably proceeds by the pathways shown in eq 9.



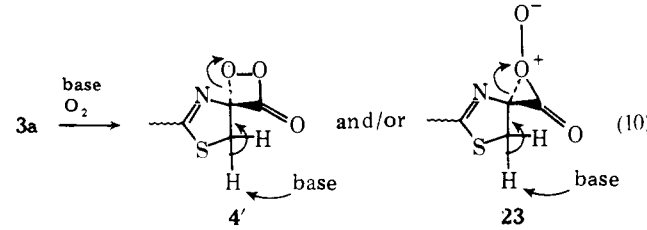
21



22

A second product of the oxidation is the methyl ester of 4-hydroxyluciferin (**10**). This compound may stem from a reduction of the hydroperoxide (**22**), possibly by the carbanion precursor **21**. Dioxetanone formation would be slow in this case because of the poor leaving-group ability of the methoxide ion, and the hydroperoxide (**22**) thus meets a different fate. Corresponding alcohol analogues of hydroperoxides have been observed in the chemiluminescence of *Cypridina* luciferin,[33] lophine,[34] indoles,[35] and tryptophans.[36]

The chemiluminescent reaction of the ethoxyvinyl ester of luciferin **3a** with oxygen in the presence of base yields much more dehydroluciferin (**7**) (as the free acid after workup, 48–73%) than does the methyl ester (17% yield as the ester), suggesting that the carboxyl group is involved in the excess production of dehydroluciferin. Possible sources of the dehydroluciferin are outlined in structures **4′** and **23** (followed by



4′ 23

reduction or hydrolysis of the peracid formed). Perepoxides have been proposed as intermediates in the addition of oxygen to ketenes,[25] the addition of singlet oxygen to hindered alkenes,[37] and in certain epoxidations.[38]

The ready formation of dehydroluciferin in the chemiluminescence of the ethoxyvinyl ester suggests that it might be formed in vivo[39] in an analogous way from luciferyl adenylate. Thus, the dehydroluciferin found in fireflies may be essential

rather than artifactual; possibly it is involved as an inhibitor in shaping the flash nature of the light emission in fireflies.[2,40]

Experimental Section

Introduction. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. NMR spectra were measured using a JEOLCO MH-100 and peaks are reported in parts per million relative to tetramethylsilane. Mass spectra were obtained with an Hitachi Perkin-Elmer RMU-6D spectrometer at 70 eV. Fluorescence and chemiluminescence spectra were determined on a Hitachi Perkin-Elmer Model MPF-2A spectrofluorimeter. High-pressure liquid chromatography was carried out with a Waters Associates M6000A pump, a U6K injector, and a Beckman LC-25 variable-wavelength detector.

Ethoxyvinyl 2-(6′-hydroxy-2′-benzothiazolyl)-5,5-dimethyl-Δ^2-thiazoline-4-carboxylate (**3b**) (5,5-dimethylluciferin ethoxyvinyl ester) and 2-(6′-hydroxy-2′-benzothiazolyl)-4-isopropylidene-Δ^2-thiazolin-5-one (**6**) were prepared as described by White, Suzuki, and Miano.[14] The following compounds were prepared by established procedures: 2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5,5-dimethyl-4-one (**5b**) (5,5-dimethyloxyluciferin),[4b] 2-(6′-hydroxy-2′-benzothiazolyl)-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (**1b**) (5,5-dimethylluciferin),[41] 2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**1a**) (firefly luciferin),[16] and 2-(6′-hydroxy-2′-benzothiazolyl)thiazole-4-carboxylic acid (**7**) (dehydroluciferin).[16] 2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-thiazolin-4-one (**5a**) (oxyluciferin) was provided by N. Suzuki.[15]

Ethoxyvinyl 2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylate (Luciferin Ethoxyvinyl Ester 3a). To a solution of 198 mg (0.707 mmol) of luciferin[16] in 20 mL of dry tetrahydrofuran (distilled from lithium aluminum hydride) under argon was added 1.00 mL (11.4 mmol) of ethoxyacetylene[42] followed by 0.70 mL of 0.031 M mercuric acetate (0.022 mmol) in dry tetrahydrofuran. After 19 h, TLC (silica gel, 1:1 ethyl acetate–benzene) showed essentially complete conversion of luciferin (R_f 0.0) to ester product (R_f 0.55). LC assay using column B (vide infra) gave a ratio of 93:7 for ester and luciferin, respectively. The reaction mixture was poured into ether, washed with saturated sodium bicarbonate and then water, dried over anhydrous sodium sulfate, and concentrated in vacuo to give 68.9 mg (28%) of the luciferin ethoxyvinyl ester as a pale yellow solid, mp >176 °C dec (with gas evolution): IR (KBr) 3.25 (br), 5.68, 6.00, 6.37, 6.76, 8.20, 8.70 μ; NMR (Me_2SO-d_6) δ 1.24 (t, J = 6 Hz, 3 H, CH_3), 3.7–4.2 (m, 6 H, CH_2), 5.67 (t, J = 8 Hz, 1 H, CH), 7.08 (dd, J = 3, 11 Hz, 1 H, aromatic), 7.43 (d, J = 3 Hz, 1 H, aromatic), 7.94 (d, J = 11 Hz, 1 H, aromatic), 10.2 (s, 1 H, OH); addition of deuterium oxide caused exchange of the phenolic proton at δ 10.2 and partial exchange (ca. 75% loss of signal) of the 4 proton at δ 5.67; UV λ_{max} (THF) 332 nm (ϵ 17 900), 272 (8220).

Anal. Calcd for $C_{15}H_{14}N_2O_4S_2$: C, 51.42; H, 4.03; N, 7.99. Found: C, 51.16; H, 4.08; N, 7.89.

Higher yields were obtained with ethyl acetate as the extraction solvent. After drying, the solution was concentrated to 2 mL at 0.05 mm and 4 mL of cyclohexane was added. Cooling, suction filtration, and drying at 0.05 mm gave a 53% yield of pale yellow, crystalline ester which was pure by TLC (R_f 0.52, yellow fluorescent); only a very faint spot representing luciferin was seen at R_f 0.0. Solutions of the ester in dichloromethane could be washed with sodium bicarbonate solutions with no detectable hydrolysis.

Chromatographic Procedures for Products Derived from Luciferin Ethoxyvinyl Ester Chemiluminescence. Product assays involved addition of a known amount of anthracene internal standard followed by high-pressure liquid chromatography (LC) using the following columns: column A, 30 cm × 3.9 mm Waters Associates Bondapak C_{18} eluting with 50% acetonitrile in water (v/v) at 0.5 mL/min flow rate; column B, a combination of column A in series with 15 cm × 3.9 mm Whatman Partisil-10 SCX with 55% acetonitrile in water (v/v) eluent at 0.5 mL/min flow rate. The ultraviolet variable wavelength detector was calibrated at 380 nm for the response of each component relative to anthracene using known mixtures. Luciferin and luciferin ethoxyvinyl ester were not detectable at 380 nm at the concentrations employed in chemiluminescence runs below; these compounds were assayed instead at 334 nm. The retention times follow: luciferin,[16] 15.3 min; dehydroluciferin,[16] 15.5 min; oxyluciferin,[15,43] 18.0 min; anthracene, 58.5 min using column A. For column B, the retention times follow: luciferin, 11.5 min; dehydroluciferin, 11.5 min; oxyluciferin, 18.7 min; luciferin ethoxyvinyl ester, 21.0 min; anthracene, 31.5 min. The identity of each peak in product mixtures was tentatively determined by comparison of the ultraviolet spectrum at stopped flow with authentic samples and also by comparison of retention time. The identities were confirmed (when noted below) by isolation using preparative LC; fractions were freeze-dried and analyzed by mass spectroscopy.

Products of Luciferin Ethoxyvinyl Ester (3a) Chemiluminescence. To a stirred solution of 1.25 mg (3.58×10^{-3} mmol) of luciferin ethoxyvinyl ester in 5 mL of dry, air-saturated dimethyl sulfoxide (distilled from sodium hydride) was added a 0.50-mL (0.26 mmol) aliquot of 0.51 M potassium phenoxide in 1:1 phenol–dimethyl sulfoxide (v/v) via a syringe. When the red chemiluminescence ceased, 0.2 mL of acetic acid was added. The mixture was concentrated to ca. 1 mL at 45 °C (0.5 mm) using a liquid nitrogen cooled cold finger. Methylene chloride was added and the suspension was washed with water and then with saturated sodium chloride to remove salts; the solution was then dried over anhydrous sodium sulfate and evaporated to dryness at 0.5 mm. The residue was dissolved in methanol and analyzed by LC using column B (vide supra). Assay gave oxyluciferin[43] (1.23×10^{-3} mmol, 34%), dehydroluciferin[16] (1.63×10^{-3} mmol, 46%), and an unidentified product at 22.5 min retention time [λ_{max} (stopped flow) 354 nm]. Each component was isolated preparatively by LC (vide supra) and the mass spectra were determined: m/e (rel intensity) for oxyluciferin 250 (14), 176 (100); for dehydroluciferin 278 (100), 234 (62), 194 (46). Examination of the M + 2 (m/e 280) peak of dehydroluciferin showed that luciferin was absent. Since luciferin and dehydroluciferin each had identical retention time by LC, the dehydroluciferin isolated preparatively was chromatographed on cellulose, eluting with 1:1 methanol–water. Luciferin was absent at R_f 0.8 and only one spot of dehydroluciferin was present at R_f 0.5 (blue fluorescent). Additional runs are given in Table I (runs 2–5), where LC assay was conducted with the crude reaction mixture without workup.

A similar run in the absence of oxygen (carried out under high vacuum) was nonchemiluminescent. LC assay (column A) showed only luciferin (100%) and no oxyluciferin. TLC on cellulose with 70% ethanol and 30% 1 M ammonium acetate eluent showed only luciferin at R_f 0.76 and no dehydroluciferin at R_f 0.50.

Methyl 2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylate (Luciferin Methyl Ester, 8). To a solution of 173 mg (0.618 mmol) of luciferin in 25 mL of tetrahydrofuran at 0 °C was added 3 mL of ca. 0.3 M ethereal diazomethane with stirring. TLC on silica gel eluting with 50% ethyl acetate in benzene (v/v) showed rapid conversion of luciferin (R_f 0.0) to R_f 0.41 yellow, fluorescent methyl ester. After 3 min, the reaction mixture was concentrated to dryness at 0.05 mm to obtain 175 mg of a tan solid, mp 190–192 °C dec. Crystallization from an acetone–cyclohexane mixture gave 124 mg (68%) of a colorless, crystalline solid, mp 192.5–193.0 °C dec: IR (KBr) 3.12 (br), 5.76, 6.38, 6.74, 6.87, 8.14 μ; NMR (acetone-d_6) δ 3.86 (d, J = 9 Hz, 2 H, CH_2), 3.87 (s, 3 H, $-CO_2CH_3$), 5.56 (t, J = 9 Hz, 1 H, CH), 7.32 (dd, J = 2, 9 Hz, 1 H, aromatic), 7.67 (d, J = 2 Hz, 1 H, aromatic), 8.13 (d, J = 9 Hz, 1 H, aromatic); UV λ_{max} (MeOH) 332 nm (ϵ 15 700), 269 (7400); MS (70 eV) m/e (rel intensity) parent 294 (20), 235 (100), 176 (16).[44]

Anal. Calcd for $C_{12}H_{10}N_2O_3S_2$: C, 48.96; H, 3.42; N, 9.52. Found: C, 49.19; H, 3.54; N, 9.36.

Methyl 2-(6′-Hydroxy-2′-benzothiazolyl)thiazole-4-carboxylate (Dehydroluciferin Methyl Ester, 9). The method was adapted from that of White et al.[4b] A solution of 90.0 mg (0.557 mmol) of 6-hydroxybenzothiazole-2-thiocarboxamide[4b] and 423 mg (2.33 mmol) of methyl bromopyruvate[45] in 10 mL of methanol was stirred for 21 h at 25 °C. TLC on silica gel with 50% ethyl acetate in benzene showed partial conversion of the thiocarboxamide [R_f 0.50 (yellow fluorescent)] to dehydroluciferin methyl ester [R_f 0.41 (blue fluorescent)]. Refluxing for 1.3 h under nitrogen yielded a suspension of a pale yellow, crystalline solid which was then filtered and washed with methanol to give 126 mg (77%) of the ester, mp 288–290 °C dec. Recrystallization from tetrahydrofuran in methanol gave a yellowish solid, mp 289–291 °C dec. IR (KBr) 3.25 (br), 5.82, 6.27, 6.36, 6.62, 6.96, 7.45, 7.82, 8.18 μ; NMR (acetone-d_6–Me_2SO-d_6) δ 4.02 (s, 3 H, CO_2CH_3), 7.30 (dd, J = 2, 9 Hz, 1 H, aromatic), 7.69 (d, J = 2 Hz, 1 H, aromatic), 8.11 (dd, J = 2, 9 Hz, 1 H, aromatic), 8.84 (s, 1 H, vinyl), and 10.15 (s, 1 H, phenolic); UV λ_{max} (MeOH) 351 nm (ϵ 22 000), 275 (10 400); MS (70 eV) m/e (rel intensity) 292 (100), 261

(22), 234 (40), 194 (16).

Anal. Calcd for $C_{12}H_8N_2O_3S_2$: C, 49.30; H, 2.76; N, 9.58. Found: C, 49.08; H, 2.87; N, 9.46.

This compound could be prepared also from reaction of dehydroluciferin with excess diazomethane in tetrahydrofuran at 0 °C followed by concentration to dryness at 0.05 mm.

Oxidation of the Methyl Ester of Luciferin (8). To an oxygen-saturated solution of 57.3 mg (0.195 mmol) of ester in 50 mL of dry tetrahydrofuran (distilled from sodium) was added ca. 0.42 mmol of a solid potassium phenoxide–phenol complex.[46] No chemiluminescence was observed. After the mixture was stirred for 3 min, TLC on silica gel with 50% ethyl acetate in benzene (v/v) eluent showed two components: R_f 0.39, blue fluorescence (dehydroluciferin methyl ester), and R_f 0.18, yellow fluorescence (4-hydroxyluciferin methyl ester). No oxyluciferin at R_f 0.62 (blue fluorescence) was observed. The reaction mixture was acidified with 2 drops of concentrated hydrochloric acid and concentrated to ca. 5 mL in vacuo. Ethyl acetate was added and the solution was washed with saturated solutions of sodium sulfite, sodium bicarbonate, and sodium chloride. After the solution was dried over anhydrous sodium sulfate, the TLC was unchanged from the above. The extract was concentrated in vacuo, silica gel was added, and the slurry was dried at 0.05 mm. The solid was poured onto a 20 × 2 cm silica gel column (Will, Grade 922) packed in benzene. The chromatography was monitored by TLC and UV. The following results were obtained taking 25-mL fractions: fractions 1–2, benzene, nil; fractions 3–7, 10% ethyl acetate in benzene, ca. 0.10 mg of blue fluorescence material (unidentified); fractions 8–9, 20% ethyl acetate in benzene, nil; fractions 10–13, 20% ethyl acetate in benzene, 9.6 mg (17% yield) of dehydroluciferin methyl ester; fraction 14, 20% ethyl acetate in benzene, 0.09 mg of a mixture of dehydroluciferin methyl ester and 4-hydroxyluciferin methyl ester; fractions 15–27, 40% ethyl acetate in benzene, 38 mg of 4-hydroxyluciferin methyl ester. The dehydroluciferin methyl ester was pure by TLC and was identified by UV, mass spectrum, and mixture melting point with an authentic sample.

The 4-hydroxyluciferin methyl ester in benzene–ethyl acetate was decolorized using Norite and the solution was concentrated to dryness. The residue was dissolved in a small amount of tetrahydrofuran. Addition of benzene induced precipitation to afford 14 mg of a pale yellow solid, mp 205–206 °C dec. A second crop was obtained of 15 mg, mp 204–206 °C dec; total yield, 29 mg (48%); IR (KBr) 3.03 (br), 5.76, 6.35, 6.72, 8.13 μ; NMR (Me_2SO-d_6) δ 3.61 (d, J = 12 Hz, 1 H, CH_2), 3.92 (s, 3 H, CO_2CH_3), 4.19 (d, J = 12 Hz, 1 H, CH_2), 7.34 (dd, J = 2, 9 Hz, aromatic), 7.68 (d, 1 H, J = 2 Hz, aromatic), 8.17 (d, 1 H, J = 9 Hz, aromatic), 10.5 (s, 1 H, phenolic OH); UV λ_{max} (MeOH) 337 nm (ϵ 16 500), 269 (6830); MS (70 eV) m/e (rel intensity) parent 310 (8), 292 (32), 251 (100), 234 (9), 207 (8), 177 (66), 176 (34).

The 4-hydroxyluciferin methyl ester was further characterized by dehydration to dehydroluciferin methyl ester. A solution of 2.95 mg (9.52×10^{-3} mmol) of hydroxy ester **10** in 5 mL of methanol with 1 drop of concentrated sulfuric acid was refluxed for 10 min. TLC on silica gel with 50% ethyl acetate in benzene showed complete conversion from the yellow fluorescent hydroxy ester (R_f 0.15) to the blue fluorescent dehydroluciferin methyl ester (R_f 0.40). The UV spectrum had changed from λ_{max} 337 nm to λ_{max} 352 nm, and the change in optical density showed 80% conversion. Upon cooling to room temperature, 1.3 mg (46%) of dehydroluciferin methyl ester was obtained as a pale yellow, crystalline solid, mp 189–191 °C.

A similar oxidation of the methyl ester of luciferin was carried out with dry dimethyl sulfoxide (distilled from sodium hydride) as the solvent. The yellow solid residue obtained after workup and concentration to dryness was chromatographed on a 20 cm × 20 cm × 0.25 mm silica gel plate (Eastman), eluting with 50% ethyl acetate in benzene (v/v). After two passes, the top band (blue-white fluorescent) gave dehydroluciferin methyl ester (12% by UV assay). The yellow band gave 38% of 4-hydroxyluciferin methyl ester (by UV assay).

Stability of 4-Hydroxyluciferin Methyl Ester (10) to Oxidation. The procedure followed that for luciferin methyl ester oxidation in tetrahydrofuran (vide supra). A solution of 1.68 mg (5.42×10^{-3} mmol) of 4-hydroxyluciferin methyl ester in 1 mL of oxygen-saturated tetrahydrofuran containing 0.024 mmol of potassium phenoxide was stirred for 10 min and acidified with 1 drop of concentrated hydrochloric acid. TLC on silica gel with 50% ethyl acetate in benzene (v/v) showed the yellow fluorescent starting material at R_f 0.18 and a faint yellow fluorescent material at the origin. Dehydroluciferin methyl ester was not present at R_f 0.40; 5×10^{-5} mmol (0.9%) would have been detected based on TLC of a known mixture. The residue after workup was chromatographed on a 8 × 1 cm silica gel (Will, Grade 922) column eluting with 30% ethyl acetate in benzene. The starting 4-hydroxyluciferin methyl ester was obtained in the second 20-mL fraction. After concentration to dryness, UV assay in methanol showed 2.72×10^{-3} mmol (50.2% recovery).

Stability of Luciferin Methyl Ester (8) to Base without Oxygen. All operations were performed on a vacuum line in order to rigorously exclude oxygen. A solution of 12.2 mg (0.0437 mmol) of luciferin methyl ester in 15 mL of tetrahydrofuran was degassed by two freeze–pump–thaw cycles. Concurrently 3 mL (0.17 mmol) of 0.056 M potassium phenoxide in tetrahydrofuran was degassed in an adjoining vessel. A side arm contained 0.1 mL of frozen (77 K) glacial acetic acid for later use. The base solution was tipped into the reaction flask, and the green-colored reaction mixture was stirred for 10 min. The acetic acid was then thawed and washed into the reaction mixture to give a colorless solution. TLC on silica gel eluting with 50% ethyl acetate in benzene showed only yellow-green fluorescent luciferin methyl ester at R_f 0.35. The λ_{max} at 332 nm was identical with that of the starting ester; the absorbance indicated a recovery of 91%.

Stability of Luciferin to Potassium Phenoxide and Oxygen. The procedure was identical with that for luciferin ethoxyvinyl ester chemiluminescent runs and employed 1.05 mg (3.75×10^{-3} mmol) of luciferin and 0.152 mmol of potassium phenoxide in 4.0 mL of oxygen-saturated dimethyl sulfoxide. The reaction was monitored by TLC on cellulose with an eluent of 70% ethanol + 30% 1 M ammonium acetate. Over a period of 1 h, only luciferin at R_f 0.67 was observed. Dehydroluciferin at R_f 0.46 was absent. Preparative TLC on a 20 cm × 20 cm × 0.5 mm cellulose plate eluting with 50% methanol in water gave recovered luciferin at R_f 0.75. A very faint blue fluorescent band at R_f 0.5 gave 1.7×10^{-5} mmol (0.4%) of dehydroluciferin by UV analysis.

Phenyl 2-(6′-Trifluoroacetoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylate. To a stirred mixture of 65 mg (0.23 mmol) of luciferin and 240 mg (2.5 mmol) of phenol was added 3 mL of trifluoroacetic anhydride at 0 °C under nitrogen. The reaction flask was allowed to reach room temperature, and then it was kept at that temperature for 1 h. The excess phenol and trifluoroacetic anhydride were removed under vacuum to give a yellow solid, mp 117–119 °C: IR (KBr) 5.56, 5.78 μ; NMR ($CDCl_3$) δ 4.48 (d, J = 5 Hz, 2 H, CH_2), 6.20 (t, J = 5 Hz, 1 H, CH), 7.91 (s, 5 H, phenyl), 8.08 (dd, J = 2, 8 Hz, 1 H, aromatic), 8.47 (d, J = 2 Hz, 1 H, aromatic), 8.80 (d, J = 8 Hz, 1 H, aromatic); MS m/e 452 (parent), 331 ($-CO_2Ph$); UV (Me_2SO) 335, 273 nm; TLC (silica gel, eluting with 50% ethyl acetate in benzene) R_f 0.70 (major component), 0.55 (possibly phenyl ester), 0.0 (minor; luciferin and/or dehydroluciferin).

General Procedure for Chemiluminescence Quantum Yield Measurements. The apparatus used to determine light yields for luciferin ethoxyvinyl ester consisted of a R446 photomultiplier, a Fluke Model 412 B power source at 600 V, and a Keithley electrometer to measure total charge (proportional to the total light emitted). The photomultiplier was corrected for its differing response vs. wavelength.[47] The chemiluminescence spectrum of luciferin ethoxyvinyl ester in dimethyl sulfoxide using potassium phenoxide as base gave λ_{max} 625 nm and was similar to that reported for other luciferin ester derivatives (vide infra).[4b] Quantum yields were obtained relative to luminol (Φ_{CL} = 0.011), a procedure used to obtain chemiluminescence quantum yields for 5,5-dimethylluciferin ethoxyvinyl ester.[6,14]

Potassium Imidazolate. Potassium amide in liquid ammonia was prepared from 9.2 g (0.24 g-atom) of potassium in 500 mL of liquid ammonia under nitrogen with a catalytic amount of ferric nitrate nonahydrate. Imidazole (17 g, 0.25 mol) (decolorized with Norite in hot benzene followed by crystallization) was added in portions over 15 min to 0.24 mol of potassium amide in 500 mL of liquid ammonia. The ammonia was evaporated under a stream of nitrogen and the remaining solid was pulverized, washed with ether, and dried under vacuum to give an off-white powder.

Effect of Oxygen Partial Pressure on the Chemiluminescence Quantum Yield of Luciferin Ethoxyvinyl Ester (3a). Each run employed 3.40×10^{-3} mmol of luciferin ethoxyvinyl ester in 5.2 mL of dimethyl sulfoxide (degassed by three freeze–pump–thaw cycles). The sample solution (5.6 mL total volume) for each determination was contained in a 26.4-mL cylindrical Pyrex cell; a 0.024-in. pinhole aperture was interposed between the sample cell and photomultiplier (in these runs, a 1P21 photomultiplier was used). Oxygen was introduced into the

evacuated cell and the pressure measured. Light was produced by adding 0.4 mL (0.14 mmol) of 0.36 M potassium phenoxide (prepared from potassium *tert*-butoxide and excess phenol) in nitrogen-saturated dimethyl sulfoxide (v/v).

Chemiluminescence Spectra. The chemiluminescence of the ethoxyvinyl ester of luciferin normally was too fast to measure with the scanning monochromator of the fluorimeter. Thus, spectra were obtained by a point-by-point plot of intensity vs. wavelength; each point was determined by adding an aliquot of potassium phenoxide by syringe to the ester and obtaining the intensity of emission at a particular wavelength. An 8.44×10^{-6} M dimethyl sulfoxide solution gave λ_{max} 625 ± 3 nm; in tetrahydrofuran the maximum was 595 ± 3 nm. In two cases, the chemiluminescent reaction was slow enough to be measured by a scanning monochromator: addition of a pellet of potassium hydroxide to a 1×10^{-5} M dimethyl sulfoxide solution of ester gave λ_{max} 625 ± 3 nm. Addition of 2 molar equiv of potassium *tert*-butoxide to a 7.03×10^{-5} M solution of ester in tetrahydrofuran gave λ_{max} 595 ± 3 nm.

Fluorescence Measurements. Corrected spectra were obtained by standard procedures.[48-50]

The Φ_{fl} was determined for 5,5-dimethyloxyluciferin (**5b**) in dimethyl sulfoxide with excess potassium imidazolate at high dilution[48] (A = 0.050 at 540 nm). The corrected fluorescence spectrum (λ_{max} 635 nm) was compared to the corrected fluorescence spectrum (λ_{max} 580 nm, Φ_{fl} = 0.69[48]) of a dilute rhodamine B standard in ethanol having an identical optical density of 0.050 at 540 nm. Two determinations gave Φ_{fl} = 0.61, 0.64 (average 0.62). As a check, the fluorescence quantum yield of rhodamine B (A_{540} = 0.050) was determined against quinine bisulfate in 1 N sulfuric acid as a standard (λ_{max} 470 nm, Φ_{fl} = 0.55[48]). The excitation wavelength for the standard was 363 nm (A = 0.055). Two determinations gave Φ_f = 0.69 and 0.71, in agreement with the literature.

Fluorescence studies with oxyluciferin (**5a**) were more qualitative in nature and the spectra were not corrected. Oxyluciferin (**5a**) was 5.03×10^{-5} M in argon-saturated dimethyl sulfoxide. For each determination, a known amount of potassium phenoxide in dimethyl sulfoxide was added. After each fluorescence spectrum, the absorption spectrum was obtained. The sample was then acidified with glacial acetic acid and the absorption spectrum redetermined; the absorption spectrum was unchanged from that prior to base addition. The volumes of base and glacial acetic acid used changed the total volume of the sample solution by only 1% at most. The results are summarized in Table III.

Calculation of Percent Carbon Dioxide Labeled with ^{18}O Using Mass Spectroscopic Data. Accurate peak intensities were obtained by slow pen recorder scans of carbon dioxide samples. All peak heights were corrected for background. The atom percent, A, of ^{18}O in carbon dioxide was obtained from

$$A = \frac{R}{2(R+1)} \times 100$$

where R = 46/44 (m/e values).[51] The percentage N of the total carbon dioxide labeled with one ^{18}O was then given by

$$N = \frac{2(A - 0.20)}{B - 0.20} \times 100$$

where 0.2 is the natural abundance of ^{18}O and B is the atom percent ^{18}O in the ^{18}O used in the chemiluminescent reactions.

Vacuum Line Isotope Runs with Luciferin Ethoxyvinyl Ester (3a). An all-glass cell with two side attachments was used and the cell was attached to the vacuum line through a series of three traps (numbered from the reaction cell). The first attachment was used for the distillation of Me_2SO (from sodium hydride) into the reaction cell. The second attachment allowed the sublimation of potassium *tert*-butoxide into a sample of phenol contained therein. After evacuation the dimethyl sulfoxide and phenol were degassed by two freeze-pump-thaw cycles. After the potassium *tert*-butoxide was sublimed further into the side arm and the attachment was resealed with a torch, dimethyl sulfoxide was distilled into this attachment until ca. 1 mL was collected; 6 mL was then distilled into the main part of the cell and cooled to −78 °C. Oxygen which had been cooled to 77 K for 2 h prior to reaction was introduced into the reaction vessel with slight warming to attain ca. 200 mm pressure. After the contents was thawed in the apparatus, the base was dissolved in the 1 mL of Me_2SO and the solution was then tipped into the reaction pot to initiate red chemiluminescence. After 0.5 min, the reaction mixture was frozen to −196 °C. With trap 1 at −107 °C (isooctane-N_2) and trap 2 at −196 °C, the oxygen was pumped out of the system. The reaction mixture was thawed and the volatiles were distilled into the trap system for 18 min while pumping. The trap system was isolated from the apparatus and the rest of the vacuum line. Trap 2 was isolated from trap 1 and its contents thawed and recondensed into the bottom tip of the trap using liquid nitrogen. Trap 2 was then warmed to −107 °C with frozen isooctane and the volatiles were distilled into trap 3 cooled to −196 °C. Trap 2 was isolated and trap 3 warmed to room temperature. From the known volume of the system and the pressure, the yield of carbon dioxide was determined. The carbon dioxide was then transferred to a bulb for mass-spectral analysis. The above procedure successfully eliminated volatile impurities such as ethyl acetate and *tert*-butyl alcohol. After the traps were cleared, acetic acid (0.2 mL) was distilled through the system into the reaction pot and the above collection procedure was performed again. Additional carbon dioxide was not obtained after acidification. The reaction mixture was then analyzed by LC using column A (vide supra). The ^{18}O used (Miles Laboratories) was 92.82 atom % enriched.

Run 6: 3.14×10^{-3} mmol of luciferin ethoxyvinyl ester; ca. 0.21 mmol of potassium phenoxide; 209 mm $^{16}O_2$ (Linde extra dry); 0.393 mm (22.0%) carbon dioxide (natural abundance); oxyluciferin, 4.94×10^{-4} mmol (15.7%); dehydroluciferin, 1.61×10^{-3} mmol (51.3%).

Run 7: 3.05×10^{-3} mmol of luciferin ethoxyvinyl ester; ca. 0.27 mmol of potassium phenoxide; 207 mm $^{18}O_2$; 0.298 mm (17.3%) carbon dioxide, R = 0.10, 9.3% ^{18}O in carbon dioxide; oxyluciferin, 5.12×10^{-4} mmol (16.8%); dehydroluciferin, 1.67×10^{-3} mmol (54.8%).

Run 11: 3.12×10^{-3} mmol of luciferin ethoxyvinyl ester; ca. 0.23 mmol of potassium phenoxide; 204 mm $^{18}O_2$; carbon dioxide not quantitated, R = 2.42, 76% ^{18}O in carbon dioxide; oxyluciferin, 4.37×10^{-4} mmol (14.0%); dehydroluciferin, 1.15×10^{-3} mmol (36.9%).

Control Run. No luciferin ethoxyvinyl ester, ca. 0.21 mmol of potassium phenoxide, 156 mm $^{18}O_2$; 0.010 mm volatile material unidentified; no volatiles obtained after acidification.

Standard Isotope Runs with 5,5-Dimethylluciferin Ethoxyvinyl Ester (3b). The apparatus consisted of a 5-mL flask with a rubber septum sealed side arm; this was connected through two U-tube traps in series to a high-vacuum line. A solution of 2.04 mg (5.40×10^{-3} mmol) of 5,5-dimethylluciferin ethoxyvinyl ester in 2.0 mL of dry dimethyl sulfoxide was degassed by two freeze-pump-thaw cycles. One atmosphere of oxygen-18 (91.6 atom % excess) was introduced. A 10 molar excess of potassium phenoxide in dimethyl sulfoxide (prepared from sublimed potassium *tert*-butoxide and 1.5 equiv of phenol) was added by syringe to give red chemiluminescence. After 5 min, the reaction vessel was evacuated and the mixture neutralized with 100% phosphoric acid[17] in dimethyl sulfoxide. Vigorous evolution of carbon dioxide was observed. With trap 1 at −78 °C and trap 2 at −196 °C, the reaction vessel was evacuated. After trap 2 was isolated and warmed to −78 °C, the volatiles were collected and analyzed by mass spectroscopy. The spectrum showed a ratio of m/e 46/m/e 44 of 0.27. The percentage of carbon dioxide labeled with ^{18}O was thus 22%. The 5,5-dimethyloxyluciferin produced was isolated by preparative TLC as described previously.[14] The mass spectrum showed m/e 278 (77-mm peak height) and 280 (104 mm) to give 60% incorporation of ^{18}O label (see section below for method of calculation.)

Isotopic Exchange in Carbon Dioxide. A solution of 7.6 mg (0.068 mmol) of freshly sublimed potassium *tert*-butoxide in 2.0 mL of dimethyl sulfoxide was degassed by two freeze-pump-thaw cycles. Carbon dioxide (0.035 mm), 22% of which contained an atom of ^{18}O, was condensed into the reaction vessel cooled to −196 °C. After thawing, the reaction vessel was evacuated and 100% phosphoric acid[17] in dimethyl sulfoxide was added by syringe. With trap 1 at −78 °C and trap 2 at −196 °C, 0.030 mm of carbon dioxide was collected in trap 2. The mass spectrum showed that only 8.5% of the carbon dioxide was labeled with ^{18}O.

Vacuum Line Isotope Runs with 5,5-Dimethylluciferin Ethoxyvinyl Ester (3b). The apparatus was similar to that used for the parent luciferin ester except that it contained an additional side arm to contain phosphoric acid. For each run a glass boat containing 5,5-dimethylluciferin ethoxyvinyl ester was introduced into the main chamber of the flame-dried apparatus under nitrogen. Potassium *tert*-butoxide (ca. 10 mg) was placed in the base arm; all openings at this stage were sealed with septa; the apparatus was partially evacuated by means of a syringe, and then the base arm was sealed with a torch. In the same

manner, 100% phosphoric acid[17] was introduced to an acid side arm which was then sealed off. A 5-mL aliquot of dimethyl sulfoxide containing sodium hydride (heated partially to form dimsyl anion) was filtered through glass wool into the side arm pot (through a sleeve) which was then sealed off with a torch while frozen. The apparatus was evacuated to 2 μ. With the dimethyl sulfoxide at −78 °C, potassium *tert*-butoxide was sublimed to the upper part of the base side arm at ca. 170 °C by heating with an oil bath; the unsublimed residue was removed by cutting off the end of the tube with a torch. After the dimethyl sulfoxide was degassed by three freeze-pump-thaw cycles, 3 mL was distilled into the reaction pot (cooled to −196 °C) while pumping. The side arm pot was then removed with a torch while keeping the apparatus evacuated. Oxygen-18 (53.5 atom % excess) was introduced. Once thawed, the ester solution was used to dissolve the base to give red chemiluminescence. After evacuation of the vessel and acidification, the volatiles were passed into trap 1 (−78 °C) and trap 2 (−196 °C). After trap 2 was isolated from trap 1 and warmed to −78 °C, the carbon dioxide was quantitated using the ideal gas law. This was collected in a bulb cooled to −196 °C for mass-spectral analysis. The results are summarized below. In run 1, 5,5-dimethyloxyluciferin was isolated by preparative cellulose TLC as described previously.[14] The mass spectrum showed *m/e* 278 (83-mm peak height) and 280 (91 mm) thus giving a calculated ^{18}O incorporation of 94%.

The data are summarized as follows: mmol luciferin ethoxyvinyl ester reactant, pressure (% yield) of carbon dioxide, ratio R = *m/e* 46/*m/e* 44, calculated percent of ^{18}O in carbon dioxide.

Run 8: 6.65×10^{-3} mmol ester, 0.055 mm (55%), R = 0.81, 83% ^{18}O in carbon dioxide.

Run 9: 1.48×10^{-2} mmol ester, 0.055 mm (28%), R = 0.57, 67% ^{18}O in carbon dioxide.

Run 10: 1.46×10^{-2} mmol ester, 0.065 mm (34%), R = 0.45, 57% ^{18}O in carbon dioxide. In this run *m/e* 46 peak height required a 10% reduction due to a contribution by contaminating dimethyl sulfide; the ratio *m/e* 62/*m/e* 46 of 0.36 (authentic material) was used to make the correction.

Control Run. No ester, 0.01 mm of volatile material, impure carbon dioxide *m/e* 48 (37%), 46 (100%), 44 (74%). The atom percent incorporation in carbon dioxide was calculated[51b] to be 42%, which gives 1.55 atoms of ^{18}O per carbon dioxide after correction for the atom percent excess in the $^{18}O_2$ used.

Analysis of [^{18}O]5,5-Dimethyloxyluciferin (5b). Mass spectra of both labeled and unlabeled 5,5-dimethyloxyluciferin were run using the solid inlet system of the mass spectrometer. The spectrum of the unlabeled sample had a molecular ion (P) at *m/e* 278, a P + 1 peak with an intensity 15.5% that of P, and a P + 2 peak with an intensity 9.3% that of P. The height of the labeled 5,5-dimethyloxyluciferin molecular ion at *m/e* 280 was corrected for the P + 2 contribution from unlabeled 5,5-dimethyloxyluciferin by multiplying the observed *m/e* 278 peak height by 0.093 and subtracting this value from the observed *m/e* 280 peak. These values were then analyzed by the following method: x is the observed peak height of *m/e* 280 corrected for the P + 2 peak of *m/e* 278, y is the peak height of *m/e* 278, and the values of x and y corrected for the atom percent oxygen-18 used are x' and y', respectively, where $x' = x/B$ and $y' = y - (x' - x)$ and B is the atom percent oxygen-18 in the oxygen-18 used. Thus the percentage of the total 5,5-dimethyloxyluciferin labeled with one atom of oxygen-18 is equal to $x'/(x' + y')100$.

Isotope Run with ^{18}O Water and ^{16}O Oxygen. The procedure for the ^{18}O isotope run was as described for the ^{18}O molecular oxygen run except for the following modifications. After the 5,5-dimethylluciferin ethoxyvinyl ester (11.8 mg, 3.12×10^{-2} mmol) was pushed into the central reaction chamber, ^{18}O water (100 μL, 81.8 atom % ^{18}O) was placed into a small piece of glass tubing which had one end sealed. The tube filled with ^{18}O water was pushed up the base side arm into the central reaction chamber. At this point the central reaction chamber was frozen in a dry ice-acetone bath until the vessel was evacuated. It was then cooled to liquid nitrogen temperatures for the sublimation of potassium *tert*-butoxide and the distillation step. Enough dimethyl sulfoxide was distilled to make the final solution ca. 1.5-2.0 M in ^{18}O water. Matheson Research Grade oxygen (analyzed <1 ppm CO_2) was used instead of oxygen-18. The carbon dioxide collected (195 μ) was analyzed by mass spectroscopy. The heights of the *m/e* 44, 46, and 48 peaks in the mass spectrum were 66, 136, and 84 mm, respectively. Analysis and correction of these data for the 81.8 atom % ^{18}O used gave the following percentages of labeled carbon dioxide: $C^{18}{}^{-18}O_2$, = 34.2%; $C^{18}{}^{-16}O_2$, 51.8%; $C^{16}{}^{-16}O_2$, 14.0%.

Acknowledgments. We thank the U.S. Public Health Service for its financial support (Grant GM 19488) and Martha Umbreit and Dr. N. Suzuki for valued assistance.

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Conformational Studies on Humulene by Means of Empirical Force Field Calculations. Role of Stable Conformers of Humulene in Biosynthetic and Chemical Reactions

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Abstract: The conformational behavior of humulene, an 11-membered, carbocyclic, biosynthetically important sesquiterpene, was studied by empirical force field calculations. Estimation of heats of formation of four strain-minimum conformers revealed, in addition to the known conformer CT, the possible presence of another comparably stable conformer, CC. The ring inversion barrier between the CT enantiomers was estimated at $\Delta H^{\ddagger} = 14.17$ kcal/mol. We suggest that the newly recognized CC conformer is responsible for the biosynthesis of hirsutanoid and bicyclohumulenone and a transannular cyclization reaction to yield bicyclohumuladiol.

Humulene is one of the fundamental compounds in sesquiterpene biosynthesis. Farnesyl pyrophosphate undergoes head-to-tail intramolecular cyclization to produce humulenic and germacrenic cations and from both of them versatile cyclic sesquiterpene skeletons are derived[1] (Figure 1). In 1966, Allen and Rogers[2] found that a tricyclic bromohydrin,[3] derived from humulene by treatment with NBS in aqueous acetone, had a conformation quite similar to that of humulene-$AgNO_3$ complex, by means of X-ray crystallographic analysis[4] (Figure 2). Since then, the conformational similarity between a reactant and the product has been discussed in several papers[5] describing humulene, germacrene, and other medium-sized cycloolefin chemistry. In the course of studies on the biogenetic-like synthesis of illudoids,[6] we were interested in the relationship among humulene conformations, its transannular in vitro reactions, and biosyntheses of humulene-derived sesquiterpenes. Roberts and his co-workers studied the conformation of humulene by means of NMR spectroscopy and estimated the ring flipping barrier. They were, however, unable to obtain information about the actual shapes of the stable conformations.[7] In the absence of any experimental clue to determine the predominant conformations of this apparently flexible molecule, we resorted to molecular mechanics calculations[8] to assess the relative stabilities of its conformers. The ring inversion barrier was also calculated by the same method. Among the many empirical force fields being available,[10] the most popular program, Allinger's "MMI",[11] was used in this work.

Results and Discussion

Inspection of the molecular model shows that, in the 11-membered ring containing three endocyclic trans double bonds, the planes of the latter should be almost perpendicular to the plane of the ring. Thus the number of stable conformers is limited to the number of combinations of the directions of the three double bonds. Four stable conformations, CT, CC, TT, and TC,[12] then can readily be envisaged (Figure 3). The CT form appears in the crystalline silver nitrate complex.[4] Noting the chirality of three double bonds, eight possible stable conformers are *RSR*-CT, *RRR*-CC, *RRS*-TC, *RSS*-TT,[13] and the four enantiomers. Each of them can be correlated with seven other conformers through single or multiple double bond plane rotation. The correlation diagram of the eight conformers is depicted in Figure 4.[14] Calculations of heats of formation for the conformers at the corners and barriers of 12 interconversion processes along each edge of this cube[15] will cover the energy surface for the conformational change of humulene.

1. Stable Conformers. Energy minimizations of the four principal conformers were successfully achieved to give their precise geometries and heats of formation. Table I summarizes heats of formation and dihedral angles of the fully relaxed basic conformers and their perspective stereodrawings are depicted

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