Synthesis and chemiluminescence of new derivatives of isoluminol

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In an attempt to improve the sensitivity of luminescent immunoassays, we have prepared some new isoluminol, 7-(*N*,*N*-dialkylamino)-5-methyl-2,3-dihydrophthalazine-1,4-diones by means of a novel procedure involving the cycloaddition of dienamines to maleic acid derivatives. These compounds are characterized by the presence of a methyl group at C-5 and give quantum yields three to five times greater than those of the most efficient isoluminols in use at present.

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Afin d'améliorer la sensibilité des dosages luminoimmunoétalonnages, nous avons préparé de nouveaux isoluminols: des *N*,*N*-dialkylamino-7 methyl-5 dihydro-2,3 phtalazinedione-1,4 par une synthèse originale qui implique la cycloaddition d'une diénamine à des dérivés de l'acide maléique. Ces produits caractérisés par un groupe methyle en position C-5 ont un rendement quantique de trois à cinq fois supérieur à celui du meilleur isoluminol actuellement utilisé.

Introduction

The chemiluminescence of cyclic hydrazides was first demonstrated by Albrecht in 1928 (1); since then the efficiency and mechanism of the process continue to raise wide interest. A considerable body of synthetic work has been carried out with the object of isolating the various factors that control light production (2–4). Several of these were early identified by Drew as substituent effects: thus, the unimpeded resonance of electron-donating groups with the system generally exerts a favorable influence as does mild steric hindrance of other substituents with adjacent carbonyls at C-1 and C-4. However, substitution of the heterocyclic moiety completely inhibits the process.

A major application of chemiluminescence to clinical analysis consists in using luminescent molecules as alternatives for radioactive and enzymatic labels in immunoassays. Indeed, luminescent immunoassay (LIA) procedures have recently been described for steroids (5–7), proteins (8, 9), and other biological compounds (10). In this respect, two factors play a major role in the sensitivity of the immunoassay: the affinity of antibodies for the tracer and the specific activity of the latter. In chemiluminescence, the quantum yield is the specific activity of the tracer and probably is the most important parameter affecting the sensitivity of LIA.

Although luminol provides a greater light yield than isoluminol, derivatives of the latter that can be coupled to proteins or steroids have been used extensively since by virtue of the foregoing rules they are more efficient than substituted luminols. Schroeder *et al.* (11) prepared several luminescent compounds and found that aminobutyl ethyl isoluminol in particular showed high efficiency. The use of such a derivative in the LIA of steroids has resulted in a procedure with a sensitivity comparable to that of radioimmunoassays (RIA) (5–7). In an attempt to further improve the sensitivity of LIA, we have prepared and characterized a series of new chemiluminescent derivatives of isoluminol. This novel synthesis involves the cycloaddition of dienamines to maleic acid derivatives and was designed to give compounds with a methyl group at the C-5 position, a feature expected to enhance the quantum efficiency.

Experimental

A. Synthesis (Fig. 1)

All melting points were taken for samples in capillary tubes with a Thomas–Hoover Apparatus and are not corrected. The uv spectra were determined on a Hewlett-Packard 8450A spectrophotometer and the ir spectra on a Beckman Model IR 4250. Nuclear magnetic resonance spectra were recorded with Varian EM360 A and XL-200 spectrophotometers using tetramethylsilane as internal standard. Mass spectra were obtained with a Hewlett-Packard 5995 A spectrophotometer. Merck silica gel $60F_{254}$ for dry column chromatography and Woelm neutral aluminium oxide activity II were used throughout. Elementary analyses were carried out by Galbraith Laboratories Inc., Knoxville, TN. Exact masses were provided by the Laboratoire de spectrométrie de masse, Université de Sherbrooke, Sherbrooke, Canada.

*2-Alkylsiloxy-4-*N*-pyrrolidyl-2,4-pentadiene (**2**a or **2**a′)*

To a solution of lithium diisopropylamine (LDA) prepared in the usual way with *n*-butyllithium (*n*-BuLi) (10 mmol, 2.7 *M* in hexane), diisopropylamine (11 mmol) and tetramethylethylenediamine (TMEDA) (12) (10 mmol) in dry tetrahydrofuran (THF) (10 mL) at 0°C under nitrogen and cooled at −78°C, were added 4-*N*-pyrrolidyl-3-penten-2-one (**1***a*) (13) (10 mmol) in dry THF (15 mL) in 15 min, and then after 2 h, a solution of chlorotrimethylsilane (TMSCl) or *t*-butylchlorodimethylsilane (TBDMSCl) (11 mmol), in the same solvent (8 mL; 45 min). The reaction mixture was allowed to warm and kept for 90 min at room temperature, evaporated, diluted with dry petroleum ether (bp 30–60°C, 70 mL), filtered under nitrogen, and evaporated. These operations were repeated and finally the residue was stirred for 3 h under vacuum (0.2 Torr). The unstable dienamine **2***a* or **2***a′* was used without further purification.

2-Trimethylsiloxy-4-*N*-pyrrolidyl-2,4-pentadiene (**2***a*); nmr ($CDCl_3$) δ 5.00 (1H, s), 3.97 (1H, s), 3.57 (1H, s) 3.00 (4H, m), 1.78 (7H, m), and 0.13 (9H, s).

2-*t*-Butyldimethylsiloxy-4-*N*-pyrrolidyl-2,4-pentadiene (**2***a′*); nmr ($CDCl_3$) δ 5.06 (1H, s), 3.86 (1H, s), 3.6 (1H, s), 3.06 (4H, m), 1.86 (7H, m), 0.99 (9H, s), and 0.16 (6H, m).

*3-Methyl-5-*N*-pyrrolidylphthalic anhydride (**4**a)*

To a solution of bromomaleic anhydride (849 mg, 4.8 mmol) in dry benzene (20 mL) was added under nitrogen freshly prepared crude dienamine **2***a* (1.3 g, 5.8 mmol) in the same solvent (20 mL) in 30 min at 5–7°C. The dark reaction mixture was allowed to warm to room temperature and after 2 h, the solvent evaporated, the crude product dissolved in $CHCl_3$, and separated by chromatography on silica gel (50 g). Elution with $CHCl_3$ gives phthalic anhydride **4***a*, yellow needles (218 mg, 19.5%) mp 222–222.5°C; ir ν_{max} (KBr) 1823, 1753, 1620, 1578, and 865 cm^{-1}; uv λ_{max} (C_2H_5OH) 212, 267, 321, and 386 nm (log ε 4.23, 4.19, 4.05, and 3.89); nmr ($CDCl_3$) δ 6.83 (1H, d, J = 2.2 Hz), 6.55 (1H, d, J = 2.2 Hz) (this doublet is not well defined: coupling with the 3-methyl), 3.42 (4H, m) (s, with irradiation at δ 2.10), 2.59 (3H, s) and 2.10 (4H, m) (s, with irradiation at δ 3.42); mass spectrum: m/e 231 (M^+). *Anal.* calcd. for $C_{13}H_{13}O_3N$: C 67.52, H 5.66, N 6.06; found: C 67.65, H 5.62, N 6.02.

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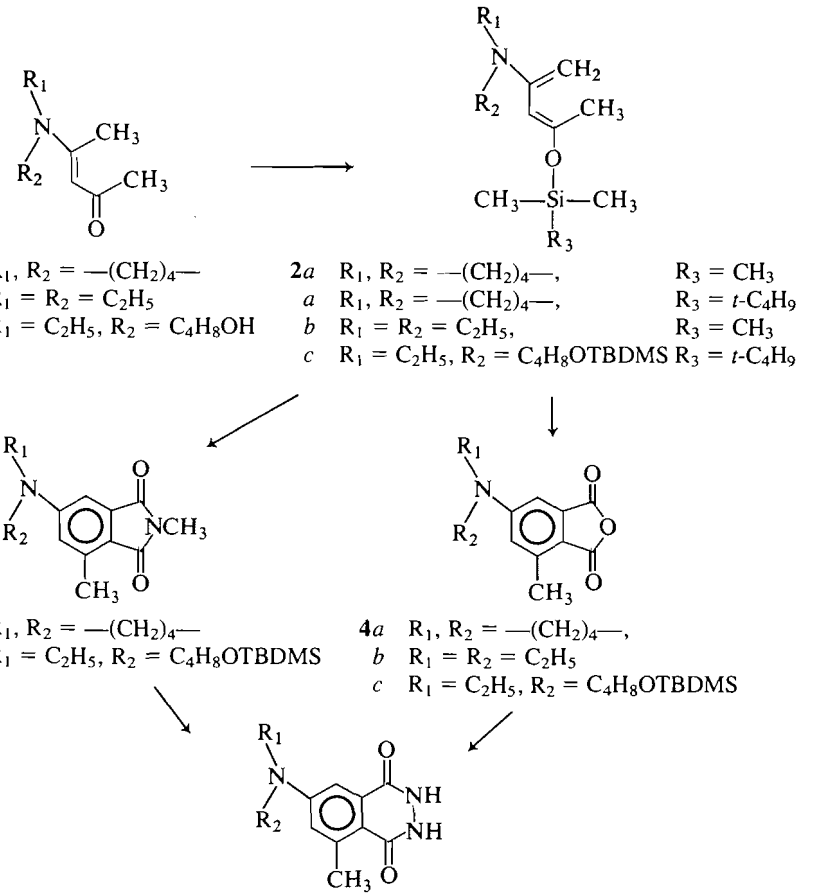

FIG. 1. Synthesis of chemiluminescent compounds.

*3-Methyl-5-*N*-pyrrolidyl-*N*-methylphthalimide (**3**a)*

To a solution of *N*-methylmaleimide (488 mg, 4.4 mmol) in dry benzene (10 mL) was added under nitrogen freshly prepared crude dienamine **2***a*′ (1.07 g, 4 mmol) in the same solvent (10 mL) in 30 min at 5–7°C. The reaction mixture was kept overnight at room temperature and then refluxed for 1 h. Palladium (10%) on activated charcoal (100 mg) was then added and refluxing continued for 3.5 h. After cooling, the catalyst was removed by filtration and the product isolated by chromatography on silica gel (100 g). Elution with CH_2Cl_2 gave methylphthalimide **3***a* (230 mg, 23.5%), yellow needles, mp 159.5–159.8°C (CH_2Cl_2, $(C_2H_5)_2O$); ir ν_{max} (KBr) 1748, 1685, 1610, 1575, and 855 cm^{-1}, uv λ_{max} (C_2H_5OH) 212, 268, 323, and 402 nm (log ϵ 4.46, 4.50, 3.93, and 3.92); nmr ($CDCl_3$) δ 6.83 (1H, d, J = 2.4 Hz), 6.38 (1H, d, J = 2.4 Hz) (this doublet is not well defined: coupling with 3-methyl), 3.39 (4H, m) (s with irradiation at δ 2.06), 3.10 (3H, s), 2.59 (3H, s), and 2.06 (4H, m) (s with irradiation at δ 3.39); $C_{14}H_{16}O_2N_2$ requires 244.1212; found 244.1206. *Anal.* calcd.: C 68.83, H 6.60, N 11.47; found: C 69.19, H 6.56, N 11.70.

*3-Methyl-5-*N,N*-dimethylaminophthalic anhydride (**4**b)*

In a similar reaction, 4-(*N*-dimethylamino)-3-penten-2-one (**1***b*) (13) (750 mg, 5 mmol) gave anhydride **4***b* (143 mg, 0.613 mmol, 12.3%), yellow needles, mp 128–128.5°C ($(C_2H_5)_2O$, petroleum ether); ir ν_{max} (KBr) 1823, 1750, 1615, 1570, and 840 cm^{-1}; uv λ_{max} (C_2H_5OH) 214, 268, 321, and 385 nm (log ϵ, 4.33, 4.31, 4.16 and 4.00); nmr ($CDCl_3$) δ 6.94 (1H, d, J = 2.2 Hz), 6.65 (1H, d of q, J = 2.2 and 0.6 Hz), 3.47 (4H, q, J = 7.3 Hz), 2.60 (3H, s), 1.24 (6H, t, J = 7.3 Hz); mass spectrum m/e 233 (M^+). *Anal.* calcd. for $C_{13}H_{15}O_3N$: C 66.93, H 6.48, N 6.00; found: C 66.89, H 6.21, N 5.91.

*7-(*N,N*-Dialkylamino)-5-methyl-2,3-dihydrophthalazine-1,4-dione (**5**a or **5**b)*

A solution of phthalic anhydride **4***a* or **4***b* (0.43 mmol) in acetic acid (4 mL) was refluxed with hydrazine hydrate (14) (85%) for 1 h under nitrogen and then poured, after cooling, into ice water. The resulting precipitate was filtered and dried: 5-methyl-7-*N*-pyrrolidyl-2,3-dihydrophthalazine-1,4-dione (**5***a*) (82 mg, 0.33 mmol, 77%), white crystals, mp 330–335°C dec. (CH_3OH, NH_4OH 10% in H_2O); ir ν_{max} (KBr) 1640, 1595, 1545, and 845 cm^{-1}, uv λ_{max} (0.1 *M* K_2CO_3) 226, 287, and 326 nm (log ϵ 4.34, 4.45, and 4.13); nmr [$(CD_3)_2SO$] δ 6.77 (1H, s), 6.75 (1H, s), 3.35 (4H, m) (under H_2O peak visible by addition of D_2O) (s with irradiation at δ 1.98), 2.72 (3H, s), 1.98 (4H, m) (s with irradiation at δ 3.35); mass spectrum m/e 245 (M^+). *Anal.* calcd. for $C_{13}H_{15}O_2N_3$: C 63.65, H 6.16, N 17.13, found: C 63.78, H 6.26, N 16.99.

This product (65 mg, 0.26 mmol, 63.4%) was also prepared under the same conditions (reflux time, 12 h) from *N*-methylphthalimide **3***a* (100 mg, 0.41 mmol).

5-Methyl-7-(*N*,*N*-dimethylamino)-2,3-dihydrophthalazine-1,4-dione (**5***b*) (93 mg, 0.37 mmol, 86%), white crystals, mp 290–292°C dec. (CH_3OH, NH_4OH 10% in H_2O); ir ν_{max} (KBr) 1635, 1595, 1540 and 845 cm^{-1}; uv λ_{max} (0.1 *M* K_2CO_3) 227, 288, and 326 nm (log ϵ 4.17, 4.43, and 4.11); nmr [$(CD_3)_2SO$] δ 6.89 (2H, s), 3.44 (4H, 1, J = 7.0 Hz), 2.72 (3H, s), 1.14 (6H, t, J = 7.0 Hz); mass spectrum m/e 247 (M^+). *Anal.* calcd. for $C_{13}H_{17}O_2N_3$: C 63.13, H 6.93, N 16.99; found: C 62.97, H 7.20, N 16.83.

*4-(*N*-ethyl-*N*-4′-hydroxybutylamino)-3-penten-2-one (**1**c)*

To 2,4-pentadione (20 g, 0.2 mol) in $CHCl_3$ (50 mL) was added

rapidly, redistilled oxalyl chloride (63 g, 0.5 mol) according to the procedure of Clark and Heathcock (15). The mixture was refluxed (45 min), evaporated, and upon distillation, the residue yielded 4-chloro-3-pentadien-2-one (15.8 g, 66%, bp 34°C/8 Torr, lit. (16) 44–45°C/23 Torr). This product (2.38 g, 20 mmol) in dry THF (10 mL) was added (5 min) to a solution of 4-(ethylamino)butanol (17, 18) (2.38 g, 20 mmol) and triethylamine (2.78 mL, 20 mmol) in the same solvent (10 mL). After 2 h at 50–55°C, an additional amount of this substance (1.19 g, 10 mmol) and triethylamine (1.4 mL) was added and the solution heated at 60°C for 1 h. After cooling, the precipitate was filtered, the solvent evaporated, and the oily residue purified by chromatography on aluminium oxide (150 g). Elution with CH_2Cl_2 and $CH_3CO_2C_2H_5$ yielded hydroxyenaminone **1***c* (2.4 g, 78%), white crystals, mp 41–41.5°C; ir ν_{max} (KBr) 3300, 1605, 1525, and 1043 cm^{-1}; uv λ_{max} (C_2H_5OH) 312 nm (log ϵ 4.38); nmr ($CDCl_3$) δ 5.09 (1H, s), 3.70 (2H, t, J = 6.0 Hz), 3.29 (5H, m), 2.52 (3H, s), 2.07 (3H, s), 1.7–1.5 (4H, m), and 1.17 (3H, t, J = 7 Hz); mass spectrum m/e 199 (M^+). *Anal.* calcd. for $C_{11}H_{21}O_2N$: C 66.29, H 10.62, N 7.03; found: C 66.02, H 10.85, N 6.93.

*2-(*t*-Butyldimethylsiloxy)-4-[*N*-4′-(*t*-butyldimethylsiloxy)butyl-*N*-ethylamino-2,4]-pentadiene (**2**c)*

To a solution of LDA prepared in the usual way from *n*-BuLi (8.8 mmol, 2.7 *M* in hexane), diisopropylamine (8.8 mmol), and TMEDA (12) (4.4 mmol) in dry THF (9 mL) at 0°C under nitrogen and then cooled to −78°C was added hydroxy enaminone **1***c* (800 mg, 4 mmol) in the same solvent (25 mL) during a period of 30 min. After 2 h, a solution of TBDMSCl (1.47 g, 9.6 mmol) in the same solvent (5 mL) was added over a period of 30 min. The reaction mixture was allowed to warm and kept for 90 min at room temperature, evaporated, diluted with dry petroleum ether (bp 30–60°C) (50 mL), filtered under nitrogen and evaporated. These operations were repeated and finally the residue was stirred for 3 h under vacuum (0.2 Torr). An nmr spectrum of the crude dienamine **2***c* (1.26 g, 2.9 mmol, 72%) was recorded, ($CDCl_3$, N_2) δ 1.9 (3H, m), 0.90 (18H, m) and 0.1 (12H, m); showing the disappearance of two singlets at δ 2.52 and 2.07. This very unstable dienamine **2***c* was used without further purification.

*5-*N*-4′-[*t*-Butyldimethylsiloxy)butyl-*N*-ethylamino]-3-methylphthalic anhydride (**4**c)*

To a solution of bromomaleic anhydride (684 mg, 3.87 mmol) in dry benzene (20 mL) was added in 30 min at 5–7°C under nitrogen freshly prepared crude dienamine **2***c* (700 mg, 1.6 mmol) from the preceding reaction in the same solvent (20 mL). The dark reaction mixture was allowed to warm to room temperature and after 15 h, the solvent was evaporated, the crude product dissolved in CH_2Cl_2, filtered, and separated by chromatography on silica gel (50 mg). Elution with CH_2Cl_2 gave phthalic anhydride **4***c*, yellow oil (28 mg, 0.072 mmol, 4.5%); ir ν_{max} (film) 1827, 1765, 1617, 1580, 1250, 1090, and 830 cm^{-1}; uv λ_{max} (C_2H_5OH) 214, 268, 322, and 387 nm (log ϵ 3.98, 3.97, 3.83, and 3.49); nmr ($CDCl_3$) δ 6.94 (1H, d, J = 1.9 Hz), 6.65 (1H, d, J = 1.9 Hz), (this doublet is not well defined: coupling with the 3-methyl), 3.67 (2H, t, J = 5.9 Hz), 3.46 (4H, m) (3.49, s and 3.42, m with irradiation at δ 1.23), 2.59 (3H, s) 1.8–1.5 (4H, m), 1.23 (3H, t, J = 7.2 Hz), 0.89 (9H, s), 0.06 (6H, s); $C_{21}H_{33}O_4NSi$ requires 391.2179; found 391.2181. *Anal.* calcd: C 64.41, H 8.49, N 3.57; found: C 64.98, H 8.56, N 3.34).

*5-[*N*-4′-(*t*-Butyldimethylsiloxy)butyl-*N*-ethylamino]-3-methyl-*N*-methylphthaleimide (**3**c)*

To a solution of *N*-methylmaleimide (286 mg, 2.58 mmol) in dry benzene (10 mL) was added in 30 min at 5–7°C under nitrogen freshly prepared crude dienamine **2***c* (1.1 g, 2.57 mmol) in the same solvent (10 mL). After 48 h at room temperature, the solvent was evaporated and palladium (10%) on activated charcoal (100 mg) with xylenes (10 mL) was added. The reaction mixture was refluxed (6 h), cooled, filtered, and then purified by chromatography on silica gel (100 g). Elution (CH_2Cl_2, $CH_3CO_2H_5$; 99:1) gave methylphthaleimide **3***c*, yellow oil (174 mg, 0.45 mmol, 17.5%), ir ν_{max} (film) 1755, 1695, 1612, 1585, 1245, 1090, and 830 cm^{-1}; uv λ_{max} (C_2H_5OH) 211, 270, 324, and 402 (log ϵ 4.06, 4.09, 3.51, and 3.51); nmr ($CDCl_3$) δ 6.88 (1H, d, J = 1.9 Hz), 6.44 (1H, d, J = 1.9 Hz) (this doublet is not well defined: coupling with the 3-methyl), 3.63 (2H, t, J = 5.8 Hz), 3.42 (2H, q, J = 7.1 Hz), 3.35 (2H, t, J = 7.6 Hz), 3.07 (3H, s), 2.55 (3H, s), 1.65–1.44 (4H, m), 1.17 (3H, t, J = 5.8 Hz), 0.86 (9H, s), 0.03 (6H, s); $C_{22}H_{36}O_3N_2Si$ requires 404.2495; found 404.2484.

*7-[*N*-4′-(*t*-Butyldimethylsiloxy)butyl-*N*-ethyl amino]-5-methyl-2,3-dihydrophthalazine-1,4-dione (**5**c)*

A solution of methylphthalimide **3***c* (47 mg, 0.12 mmol) in acetic acid (1 mL) was refluxed with hydrazine hydrate (14, 19) (85%) (2 mL) under nitrogen for 4 h. After cooling, precipitated dihydrophthalazinedione **5***c* (32 mg, 0.079 mmol, 66%) was isolated by filtration (this product (16 mg, 0.039 mmol, 64%) was also prepared from phthalic anhydride **4***c* (24 mg, 0.061 mmol)), white crystals, mp 107–108.5°C (CH_3COCH_3, hexanes); ir ν_{max} (KBr) 1650, 1592, 1250, 1100, and 830 cm^{-1}; uvλ_{max} (0.1 *M* K_2CO_3, C_2H_5OH 4:1) 224, 289, and 327 nm (log ϵ 4.24, 4.47, and 4.15); nmr ($CDCl_3$) δ 7.25 (1H, m), 6.86 (1H, m), 3.75 (2H, t, J = 6.0 Hz), 3.52–3.40 (4H, m), 2.87 (3H, s), 1.8–1.5 (4H, m), 1.24 (3H, t, J = 6.0 Hz), 0.9 (9H, s) and 0.065 (6H, s); $C_{21}H_{35}O_3N_3Si$ requires 405.2448; found 405.2444. *Anal.* calcd.: C 62.18, H 8.49, N 10.35; found: C 62.18, H 8.69, N 10.36.

*7-[*N*-Ethyl-*N*-4′-hydroxybutylamino]-5-methyl-2,3-dihydrophthalazine-1,4-dione (**5**d)*

Methylphthalimide **3***c* (56 mg, 0.144 mmol) was refluxed with hydrazine hydrate (19, 20) (85%, 1 mL) under nitrogen for 1 h. The solvent was evaporated, the product dried overnight under vacuum (0.2 Torr) over P_2O_5 and then dissolved in a dry THF (5 mL) solution of tetrabutylammonium fluoride (21) (0.432 mmol, 1 *M* in THF). The reaction mixture was stirred at room temperature (3 h), then evaporated; H_2O (10 mL) added and alcohol **5***d* isolated by filtration (17 mg, 0.058 mmol, 40.6%), light yellow crystals, mp 221–222°C (CH_3OH, CH_3COCH_3); ir ν_{max} (KBr) 3260, 1640, 1592, 1020, and 840 cm^{-1}; uv λ_{max} (0.1 *M* K_2CO_3) 227, 288, and 326 nm (log ϵ 4.18, 4.44, and 4.12); nmr [$(CD_3)_2SO$] δ 6.86 (2H, s), 4.45 (1H, broad, exchange with D_2O), 3.40 (6H, m) (under H_2O, visible by addition of D_2O), 2.69 (3H, s), 1.63–1.4 (4H, m) and 1.10 (3H, t, J = 6.7 Hz). *Anal.* calcd. for $C_{15}H_{21}O_3N_3$: C 61.83, H 7.27, N 14.42; found: C 61.82, H 7.38, N 14.42.

B. Chemiluminescence

Measurements of luminescence were made on a LKB-Wallac 1251 luminometer using the automatic mode for injection and reading of the decay portion of light from the 3rd second to the 12th. Aliquots of the luminescent compound in 100 μL of phosphate buffer (0.01 *M* phosphate-buffered saline, pH 7.4) were introduced into the cuvettes (Clinicon, polystyrene 2174-086) and volumes were completed with 200 μL of NaOH (0.03 *N* to 2.0 *N*) according to the desired pH. Fixed amounts of microperoxidase (MP-11) (42 μL, 0.4 n*M*) and hydrogen peroxide (33 μL, 0.6%) were then automatically injected and light emitted was recorded.

Results and discussion

A. Synthesis

Early work on cyclic hydrazides involved mainly the search for structure-efficiency relationships while more recent studies have concentrated on the mechanism of the luminescence reaction (2, 22, 23) and on applications of these molecules as potential tracers for biochemical analysis (5–10). Synthesis of luminescent compounds has been carried out starting with nitrophthalic acid derivatives (2, 3, 19, 20) but substituted luminols or isoluminols have only been obtained by tedious step-wise processes (10, 11). In the present approach, 5-alkyl isoluminols are formed by a simple and general procedure in which all substituents are introduced in a single step, the Diels–Alder addition of a 2-amino-4-siloxydiene to bromomaleic anhydride. The required diene **2** has been prepared from the corresponding tertiary enaminone **1** using the observation that silylation of enaminones though (24) easy in the case of primary ones, is more difficult with secondary and quite un-

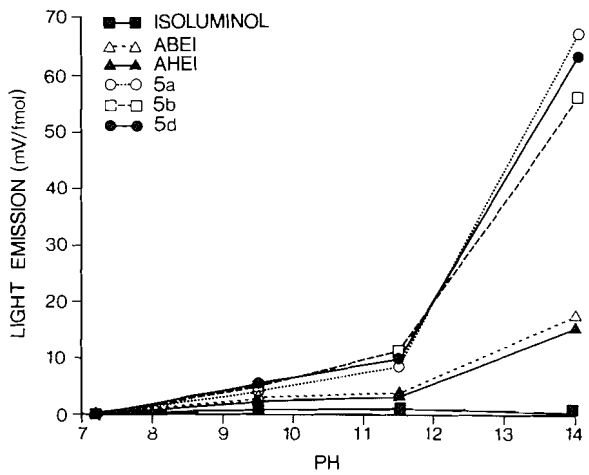

FIG. 2. Effect of pH on the decay portion (3rd to 12th second) light emission produced by isoluminol and its derivatives (ABEI, aminobutylethylisoluminol; AHEI, aminohexylethylisoluminol; **5***a*, **5***b*, and **5***c*, see Experimental).

known for tertiary examples. It was found (25) that strong base (LDA) was necessary for the regiospecific (and probably kinetic) removal of the hydrogen in the γ-position of enaminone **1** while the resulting enolate could readily be trapped as the enol silyl ether in the usual way (**1***a* and **1***b* are well described compounds; **1***c* can be prepared conveniently from 4-ethylaminobutanol (15, 17) and 4-chloro-3-pentene-2-one).

All of the dienes obtained are very labile particularly those containing a trimethylsilyl group; indeed hydroxyenaminone **1***c* does not give a detectable amount of the trimethylsiloxydiene. The crude dienol ethers were immediately added to bromomaleic anhydride or to *N*-methylmaleimide in benzene at 5–10°C. The corresponding 3-methylphthalic anhydrides were then obtained by rapid aromatization of the crude product on a column of silica gel. Use of the less reactive dienophiles *N*-methylmaleimide affords good yields of 3-methyl-*N*-methylphthaleimides **3** (especially in the case of the siloxy compound **3***c* where the yield improves by about 4-fold). In this approach, aromatization of the adduct requires an extra step involving dehydrogenation with 10% palladized charcoal and is followed by chromatography on silica gel. The pyridazine ring is formed by the well-documented action of hydrazine hydrate on anhydride **4** or *N*-methylimide **3**. In the instance of siloxyphthalazinedione **5***c* the *tert*-butyldimethylsiloxy group is best removed by fluoride ion at room temperature rather than by treatment with a refluxing mixture of acetic acid and water.

B. Chemiluminescence

With chemiluminescent substances of this type, it is a well-established fact that the pH of the medium plays an important role on the detection limit (5). Hence, we have first studied the effect of this parameter on the decay portion of the light produced by our new derivatives of isoluminol and have compared the results with those obtained for well known analogues. As in previous cases (5), the new derivatives show a clear dependency of light yield on pH (Fig. 1). This figure also indicates that the introduction of a methyl group at the C-5 position in derivatives **5***a*, **5***b*, and **5***d* also brings about a 3- to 5-fold increase on the efficiency of the chemiluminescent process.

These data extend and amplify previous observations by Brundrett *et al.* (2, 3) who had earlier shown that the presence of a methyl group at the C-5 position of luminol increases the efficiency of light production. Since the methyl groups show a weakly electron-donating character, the enhanced luminescence observed is probably accounted for by the *ortho* effect between it and the adjacent carbonyl (2, 3). Furthermore, the presence of terminal hydroxyl or amine groups seems to provide little or no influence on chemiluminescence since the three new derivatives all show approximately the same high yields.

TABLE 1. Luminescence reaction kinetics of isoluminol and its derivatives at pH 14

Compounds*	Percentage of light emitted between 0–2 s	3–12 s	13–60 s
Isoluminol	26	55	19
ABEI	21	55	24
AHEI	22	56	22
5*a*	25	60	15
5*b*	25	57	18
5*d*	22	56	22

*ABEI, aminobutylethylisoluminol; AHEI, aminohexylethylisoluminol.

Table 1 indicates the pattern of light emission of isoluminol and its derivatives. It can be seen that the increase of luminescence obtained with the new derivatives is not due to a change in the kinetics of the oxidation reaction since, as observed for the previous compounds, approximately 55% of light occurs between 3rd and 12th second at pH 14. These data confirm the high efficiency achieved when a methyl group is added.

Aminobutyl isoluminol, which shows a high level of light emission, has been extensively used in immunoassays using a luminescent tracer. The present report describes new derivatives of isoluminol which exhibit particularly high luminescent efficiency and, moreover, possess functional groups that permit ready coupling with steroids and proteins, for example with human chorionic gonadotropin; particulars will be communicated later. Hence, these compounds present an excellent potential for use as tracers in immunoassays.

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