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Chemiluminescent Products of Reaction between α-Keto Acids and 4,5-Diaminophthalhydrazide

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The chemiluminescent products formed from α-keto acids and 4,5-diaminophthalhydrazide dihydrochloride were characterized as corresponding 3-substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione. The chemiluminescence properties of the products are described.

Keywords 4,5-diaminophthalhydrazide; α-keto acid; chemiluminescent product; 3-substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione; chemiluminescence property

We previously reported a sensitive and selective chemiluminescence derivatization method[1] for the determination of α-keto acids, based on their reactions with 4,5-diaminophthalhydrazide dihydrochloride (DPH) in a dilute hydrochloric acid solution. The present paper describes the isolation and characterization of the chemiluminescent (CL) compounds produced from 6 kinds of α-keto acids of biological importance.

Experimental

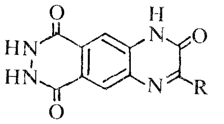
Reagents All chemicals were of the highest purity available and were used as received. Distilled water, purified with a Milli-Q II system, was used for all aqueous solutions. Sodium salts of α-ketovaleric acid (KV), α-ketoisovaleric acid (KIV), α-keto-β-methylvaleric acid (KMV), α-ketocaproic acid (KC), α-ketoisocaproic acid (KIC) and phenylpyruvic acid (PP) were purchased from Sigma (St. Louis, Mo., U.S.A.). DPH was prepared as described previously.[1,2]

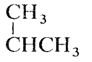
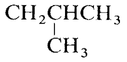
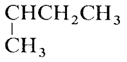
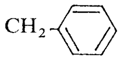
Apparatus CL intensities were measured with a Laboscience TD-4000 lumiphotometer. Ultraviolet (UV) spectra and absorbances were taken with a Hitachi 200-20 spectrophotometer in ethanol in 10 × 10-mm quartz cells. Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650—60 spectrofluorimeter in 10 × 10 mm-quartz cells; spectral bandwidth of 5 nm were used in both the excitation and emission monochromators. Infrared (IR) spectra were recorded with a JASCO IR-810 spectrophotometer in KBr pellets. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a Hitachi R-90 using approximately 2% (w/v) solutions in dimethyl-d_6 sulfoxide with tetramethylsilane as an internal standard. Splitting patterns were designated as follows: s, singlet; d, doublet; t, triplet; se, sextet; m, multiplet; br, broad. Fast atom bombardment mass spectra (MS) were taken with a JEOL DX-300 spectrometer. Uncorrected melting points were measured with a Gallen Kamp melting point apparatus.

Isolation of the Chemiluminescent Products from α-Keto Acids DPH (1.6 mmol) and an α-keto acid (0.8 mmol) were dissolved in 20 ml of a mixture of ethanol and 2.0 M hydrochloric acid containing 1.2 M β-mercaptoethanol (1 : 1, v/v). The resulting mixture was heated for 2 h in a boiling water bath. The precipitates, which were produced during the heating, were filtered off, washed with ethanol and then dried in vacuo to give the corresponding products I—VI (Table I). Yield: 45—50% for all the compounds.

Procedure for Examining Chemiluminescence Property Solutions of products I—VI (2×10^{-8} M) were prepared in water. Hydrogen peroxide (5—50 mM) and potassium hexacyanoferrate(III) (10—50 mM) solutions were prepared in water and sodium hydroxide (0.5—3.0 M) solutions, respectively. A 100-μl portion of the product solution was mixed with 100 μl of the hydrogen peroxide solution in a polystyrene tube (65 × 8 mm, i.d.). The CL reaction was initiated by automatic injection of 100 μl of the potassium hexacyanoferrate(III) solution into the tube. CL intensities were monitored immediately after the injection. Integrated CL intensity (a run time of 120 s) was used for data on the CL properties.

TABLE I. 3-Substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione



Compound No.	α-Keto acid[a]	R	mp (°C) (dec.)	Formula	Analysis (%) Calcd (Found) C	H	N
I	KV	$CH_2CH_2CH_3$	350	$C_{13}H_{12}N_4O_3$	57.35 (57.08	4.44 4.46	20.58 20.37)
II	KIV	 $\begin{array}{l} CH_3 \\ \vert \\ CHCH_3 \end{array}$	355	$C_{13}H_{12}N_4O_3$	57.35 (57.29	4.44 4.36	20.58 20.70)
III	KC	$CH_2CH_2CH_2CH_3$	355	$C_{14}H_{14}N_4O_3$	58.74 (58.64	4.93 4.98	19.57 19.82)
IV	KIC	 $\begin{array}{l} CH_2CHCH_3 \\ \quad\ \vert \\ \quad CH_3 \end{array}$	340	$C_{14}H_{14}N_4O_3$	58.74 (58.74	4.93 4.88	19.57 19.76)
V	KMV	 $\begin{array}{l} CHCH_2CH_3 \\ \vert \\ CH_3 \end{array}$	345	$C_{14}H_{14}N_4O_3$	58.74 (57.92	4.93 5.07	19.57 19.27)
VI	PP		352	$C_{17}H_{12}N_4O_3$	63.77 (63.75	3.97 3.78	17.63 17.49)

a) Abbreviations for α-keto acids; see Experimental.

Results and Discussion

Structure of the Products The reaction products of α-keto acids with *o*-phenylenediamine or 1,2-diamino-4,5-dimethoxybenzene have been characterized as 3-substituted-2(1*H*)-quinoxalinone[3] or 3-substituted-6,7-dimethoxy-2(1*H*)-quinoxalinone,[4] respectively. Accordingly, compounds I—VI should be 3-substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione; these identifications were confirmed by the analytical and spectral data shown in Tables I—III.

Chemiluminescence Properties of the Reaction Products Products I—VI generated the CL immediately after the injection of potassium hexacyanoferrate(III) in an alkaline (sodium hydroxide) solution.

The time course of the CL reaction is shown in Fig. 1. The maximum intensity was obtained at ≦1 s after the injection for all the products; the intensity at 120 s after the injection was *ca.* 1/100 of that at 1 s after the injection. The pattern of the curve was not affected by the concentrations of hydrogen peroxide, potassium hexacyanoferrate(III) and sodium hydroxide. However, the concentrations of these three reagents influenced the integrated CL intensity. The concentrations of these reagents were varied one at a time to establish the maximum integrated intensity obtainable (Fig. 2); 10 mM hydrogen peroxide, 25 mM potassium hexacyanoferrate (III) and 1.0 M sodium hydroxide gave the maximum intensity.

Table II. UV, IR and MS Data for 3-Substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione

Compound No.	UV λ_{max}^{EtOH} nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	MS *m/z* (M+1)
I	251 (5.42) 347 (4.65)	3100 (lactam, NH) 1670 (lactam, C=O)	273
II	251 (5.42) 347 (4.60)	3170 (lactam, NH) 1670 (lactam, C=O)	273
III	251 (5.42) 348 (4.63)	3100 (lactam, NH) 1670 (lactam, C=O)	287
IV	251 (5.42) 347 (4.61)	3105 (lactam, NH) 1670 (lactam, C=O)	287
V	251 (5.42) 348 (4.60)	3175 (lactam, NH) 1660 (lactam, C=O)	287
VI	251 (5.42) 350 (4.82)	3100 (lactam, NH) 1672 (lactam, C=O)	321

The integrated intensity varied slightly depending on the products (Table IV). The variation of the integrated CL intensity was examined by repeated measurement ($n=10$) using 5×10^{-8} M and 1×10^{-9} M solutions of product I; the relative standard deviations (RSDs) were 2.3 and 7.8%, respectively. The RSD of the blank was 1.1% ($n=8$). The

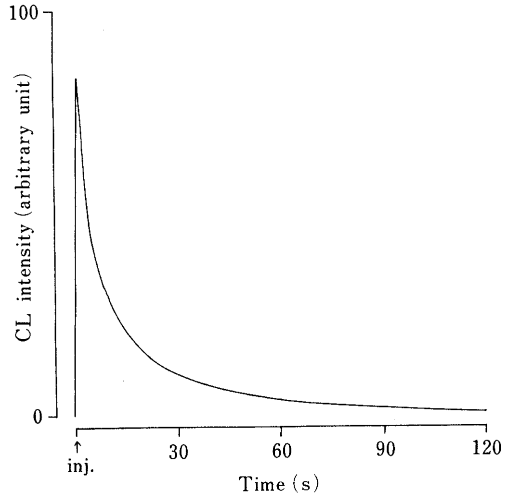
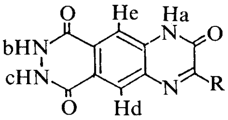


Fig. 1. Time Course of the Chemiluminescence Reaction

A portion (100 μl) of product I (2×10^{-8} M) was treated according to the procedure.

Table III. ^{1}H-NMR Data for 3-Substituted-7,8-dihydropyridazino[4,5-*g*]quinoxaline-2,6,9(1*H*)-trione



Compound No.	R	Chemical shift (ppm) H$_a$	H$_b$ and H$_c$	H$_d$	H$_e$	H$_f$	H$_g$	H$_h$	H$_i$
I	$\underset{f}{CH_2}\underset{g}{CH_2}\underset{h}{CH_3}$	12.63 (1H, br)	11.49 (1H×2, br)	8.27 (1H, s)	7.85 (1H, s)	2.82 (2H, t, *J*=7)	1.77 (2H, se, *J*=7)	0.99 (3H, t, *J*=7)	
II	$\overset{f}{CH}\overset{g}{CH_3}$ $\underset{h}{CH_3}$	12.71 (1H, br)	11.40 (1H×2, br)	8.30 (1H, s)	7.88 (1H, s)	3.24—3.65 (1H, m)	1.27 (3H×2, d, *J*=7)		
III	$\overset{f}{CH_2}\overset{g}{CH_2}\overset{h}{CH_2}\overset{i}{CH_3}$	12.65 (1H, br)	11.08 (1H×2, br)	8.27 (1H, s)	7.86 (1H, s)	2.85 (2H, t, *J*=7)	1.23—1.88 (2H×2, m)		0.94 (3H, t, *J*=7)
IV	$\overset{f}{CH_2}\overset{g}{CH}\overset{h}{CH_3}$ $\underset{i}{CH_3}$	12.71 (1H, br)	11.54 (1H×2, br)	8.28 (1H, s)	7.86 (1H, s)	2.74 (2H, d, *J*=7)	2.20—2.55 (1H, m)	0.97 (3H×2, d, *J*=7)	
V	$\overset{f}{CH}\overset{g}{CH_2}\overset{i}{CH_3}$ $\underset{h}{CH_3}$	12.69 (1H, br)	11.56 (1H×2, br)	8.30 (1H, s)	7.88 (1H, s)	1.40—2.06 (3H, m)		1.23 (3H, d, *J*=7)	0.90 (3H, t, *J*=7)
VI	$\underset{f}{CH_2}\text{-}\underset{g}{Ph(H5)}$	12.78 (1H, br)	11.46 (1H×2, br)	8.27 (1H, s)	7.86 (1H, s)	4.19 (2H, s)	7.11—7.69 (5H, m)		

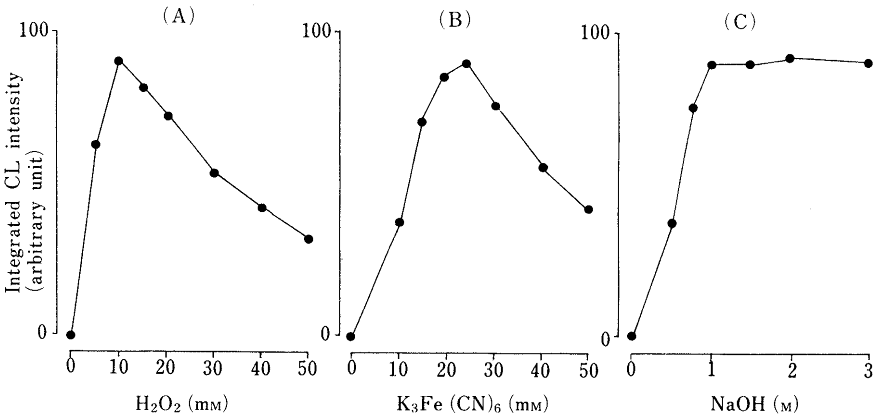


Fig. 2. Effects of (A) Hydrogen Peroxide, (B) Potassium Hexacyanoferrate(III) and (C) Sodium Hydroxide Concentrations on Integrated Chemiluminescence Intensities

Portions (100 μl) of product I (2×10^{-8} M) were tested as in the procedure at various concentrations of the reagents.

TABLE IV. Relative Chemiluminescence Intensities (RCI) and Lower Limits of Detection (LOD) of Compounds I—VI

Compound No.[a]	RCI[b]	LOD[c] (fmol/tube)
I	100	9
II	93	10
III	73	12
IV	92	10
V	150	6
VI	130	7

a) Portions (0.1 ml) of the product solution were treated as in the procedure described in Experimental. b) Integrated CL intensity of compound I was taken as 100. c) Lower limit of detection is defined as the amount in the reaction tube (0.3 ml) giving a signal/noise ratio of three.

TABLE V. Fluorescence Excitation (Ex) and Emission (Em) Maxima and Relative Intensities (RFI) of Compounds I—VI after the Chemiluminescence Reaction[a]

Compound No.	Ex (nm)	Em (nm)	RFI[b]
I	364	448	100.0
II	365	448	49.2
III	366	449	51.4
IV	365	448	64.0
V	367	448	91.1
VI	369	452	90.5

a) The maxima and intensities were measured in the reaction mixture at 1 h after the CL reaction. b) The intensity of compound I was taken as 100.

detection limit was defined as the concentration giving a CL intensity of thrice the blank, considering their RSDs. The products were detectable at 6—12 fmol/tube (6—12 × 10^{-11} M in sample solution) (Table IV) under the CL reaction conditions, which gave the maximum integrated intensity; linear relationships were observed between the ratios of the integrated intensities and the concentrations of the products in the range of 1×10^{-10}—5×10^{-7} M.

In the CL of luminol, the aminophthalate ion produced during the CL reaction has been proven to be a light-emitting species.[5,6] Therefore, 3-substituted-1,2-dihydro-2-oxoquinoxaline-6,7-dicarboxylic acid, which occurred in the reaction mixture, is expected to emit light in the case of the present study. Table V shows the fluorescence excitation and emission maxima and the relative intensities of compounds I—VI after the reaction. The emission spectra of the compounds are estimated to reach their maxima at around 448—452 nm.

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