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Fluorescent Products of Reaction between α-Keto Acids and 1,2-Diamino-4,5-dimethoxybenzene

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The fluorescent products formed from α-keto acids and 1,2-diamino-4,5-dimethoxybenzene were shown to be the corresponding 3-substituted 6,7-dimethoxy-2(1*H*)-quinoxalinones. The fluorescence properties of the products are described; they fluoresce most intensely at pH 6.0—8.0.

Keywords——1,2-diamino-4,5-dimethoxybenzene; α-keto acid; fluorescent product; 3-substituted 6,7-dimethoxy-2(1*H*)-quinoxalinone; fluorescence property

A sensitive and selective fluorimetric method for the determination of α-keto acids, based on their reaction with 1,2-diamino-4,5-dimethoxybenzene (DDB) monohydrochloride in dilute hydrochloric acid solution, has been reported.[1] The present paper describes the isolation and structural analyses of the fluorescent compounds produced from 10 kinds of α-keto acids of biological importance. Data on the fluorescence properties (fluorescence excitation and emission maxima, relative fluorescence intensity and quantum yield) of the compounds in 0.04 M phosphate buffer (pH 7.0) and methanol are also reported.

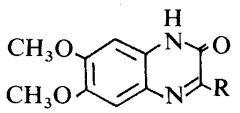
Experimental

Reagents——All chemicals were of reagent grade unless otherwise described. Double-distilled water was used. Sodium salts of pyruvic acid (PY), α-ketobutyric acid (KB), α-ketovaleric acid (KV), α-ketoisovaleric acid (KIV), α-ketocaproic acid (KC), α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoglutaric acid (KG), phenylpyruvic acid (PP) and *p*-hydroxyphenylpyruvic acid (HPP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DDB·HCl was prepared as described previously;[2] it is now available from Dojindo Laboratories (Kumamoto, Japan).

Apparatus——Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter in 10 × 10-mm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. Quantum yields were obtained by the method of Parker and Rees[3] on the spectrofluorimeter. Ultraviolet (UV) spectra were taken with a Hitachi 200-20 spectrophotometer in CH_3OH solution in 10-mm quartz cells. Infrared (IR) spectra were recorded with a Shimadzu 430 spectrophotometer in KBr pellets. ^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained with Hitachi R-22 and JEOL FX-100 spectrometers at 90 and 25.1 MHz, respectively, using approximately 10% (w/v) solutions in dimethylsulfoxide-d_6 with tetramethylsilane as an internal standard. Splitting patterns were designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; se, sextet; m, multiplet; br, broad. Values of coupling constants (J) were reported in Hz. In ^{13}C-NMR spectra, signals were assigned by both the complete decoupling and the off-resonance decoupling techniques. Electron impact mass spectra (MS) were taken with a JEOL DX-300 spectrometer. pH was measured with a Hitachi-Horiba M-7 meter at 25 °C. Uncorrected melting points were measured with a Yazawa melting point apparatus.

Isolation of the Fluorescent Products from α-Keto Acids——DDB·HCl (2.5 mmol) and an α-keto acid (2.4 mmol) were dissolved in 30 ml of 0.5 M HCl, and the mixture was heated for 2 h in a boiling water bath. The precipitates that separated on cooling the mixture in ice-water were filtered off, washed with water, dried *in vacuo* and then recrystallized from aqueous 90% (v/v) methanol to give the corresponding product (I—X) (Table I).

TABLE I. 3-Substituted 6,7-Dimethoxy-2(1*H*)-quinoxalinones



Compound No.	α-Keto acid[a]	R	Yield (%)	Appearance[b]	mp (°C)	Formula	Analysis (%) Calcd (Found)		
							C	H	N
I	PY	CH_3	91	YN	255	$C_{11}H_{12}N_2O_3$	59.99 (59.52)	5.49 5.62	12.72 12.40)
II	KB	CH_2CH_3	72	PYN	243	$C_{12}H_{14}N_2O_3$	61.53 (61.38)	6.02 6.07	11.96 11.95)
III	KV	$CH_2CH_2CH_3$	74	YN	234	$C_{13}H_{16}N_2O_3$	62.89 (62.61)	6.50 6.65	11.28 11.25)
IV	KIV	$CH(CH_3)_2$	54	CN	260	$C_{13}H_{16}N_2O_3$	62.89 (62.82)	6.50 6.74	11.28 11.18)
V	KC	$CH_2CH_2CH_2CH_3$	69	PYN	212	$C_{14}H_{18}N_2O_3$	64.11 (64.36)	6.92 7.12	10.68 10.40)
VI	KIC	$CH_2CH(CH_3)_2$	54	PYN	225	$C_{14}H_{18}N_2O_3$	64.11 (63.72)	6.92 7.14	10.68 10.38)
VII	KMV	$CH(CH_3)CH_2CH_3$	67	CN	235	$C_{14}H_{18}N_2O_3$	64.11 (64.26)	6.92 7.09	10.68 10.71)
VIII	KG	CH_2CH_2COOH	80	YN	240	$C_{13}H_{14}N_2O_5$	56.11 (56.16)	5.07 5.05	10.07 9.88)
IX	PP	CH_2–C$_6$H$_5$	68	PYN	255	$C_{17}H_{16}N_2O_3$	68.91 (68.58)	5.44 5.42	9.45 9.00)
X	HPP	CH_2–C$_6$H$_4$–OH	64	CN	262	$C_{17}H_{16}N_2O_4$	65.38 (65.69)	5.16 5.32	8.97 8.61)

a) Abbreviations for α-keto acids; see Experimental. *b*) C, colorless; P, pale; Y, yellow; N, needles.

Results and Discussion

Structure of the Products

Since the fluorescent products in the reaction of *o*-phenylenediamine with α-keto acids have been characterized as 3-substituted 2(1*H*)-quinoxalinones,[4] compounds I—X should be 3-substituted 6,7-dimethoxy-2(1*H*)-quinoxalinones.

The presence of a lactam moiety in the molecule was indicated by the IR absorption bands due to imino and carboxyl groups at 3400—3450 and 1640—1670 cm^{-1}, respectively (Table II). The ^1H- and ^{13}C-NMR and IR spectral patterns (Tables II—IV) indicated that all the compounds have the 6,7-dimethoxy-2(1*H*)-quinoxalinone structure.

From these observations, and the elemental (Table I) and mass spectral analyses (Table II), we concluded that the fluorescent product of a given α-keto acid is the corresponding 3-substituted 6,7-dimethoxy-2(1*H*)-quinoxalinone (Table I).

Fluorescence Properties of the Reaction Products

The fluorescence properties of compounds I—X in 0.04 M phosphate buffer (pH 7.0) and methanol are shown in Table V. The excitation and emission spectra of these compounds were almost identical with those obtained in the procedure for the determination of the corresponding α-keto acids.[1] Therefore, compounds I—X are the fluorescent products in the determination of the corresponding α-keto acids. The most intense fluorescence of compounds

TABLE II. UV, IR and MS Data for 3-Substituted 6,7-Dimethoxy-2(1*H*)-quinoxalinones

Compound No.	UV $\lambda_{max}^{CH_3OH}$ nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	MS *m/z* (M^+)
I	240 (4.32)	3450 (lactam, NH)	220
	294 (3.58)	1670 (lactam, C=O)	(Base peak)
	363 (4.09)	1630 (aromatic C=N and/or C=C)	
II	240 (4.32)	3420 (lactam, NH)	234
	294 (3.58)	1640 (lactam, C=O aromatic	(Base peak)
	362 (4.11)	C=N and/or C=C)	
III	240 (4.31)	3430 (lactam, NH)	248
	294 (3.59)	1660 (lactam, C=O)	(Base peak)
	362 (4.10)	1630 (aromatic, C=N and/or C=C)	
IV	240 (4.31)	3430 (lactam, NH)	248
	294 (3.58)	1655 (lactam, C=O)	(Base peak)
	361 (4.12)	1625 (aromatic C=N and/or C=C)	
V	240 (4.31)	3430 (lactam, NH)	262
	295 (3.59)	1655 (lactam, C=O)	
	363 (4.11)	1625 (aromatic C=N and/or C=C)	
VI	240 (4.28)	3430 (lactam, NH)	262
	296 (3.54)	1645 (lactam, C=O)	
	364 (4.09)	1630 (aromatic C=N and/or C=C)	
VII	240 (4.31)	3420 (lactam, NH)	262
	294 (3.58)	1645 (lactam, C=O)	(Base peak)
	363 (4.12)	1625 (aromatic C=N and/or C=C)	
VIII	240 (4.30)	3400 (lactam, NH)	278
	294 (3.56)	2930—2650 (carboxyric acid, OH)	(Base peak)
	360 (4.10)	1715 (carboxyric acid, C=O)	
		1640 (lactam, C=O)	
IX	241 (4.32)	3420 (lactam, NH)	296
	298 (3.64)	1655 (lactam, C=O)	(Base peak)
	368 (4.14)	1625 (aromatic C=N and/or C=C)	
X	240 (4.31)	3450 (lactam, NH and/or	312
	276 (3.66)	phenol OH)	(Base peak)
	287 (3.68)	1645 (lactam, C=O)	
	367 (4.15)	1615 (aromatic C=N and/or C=C)	

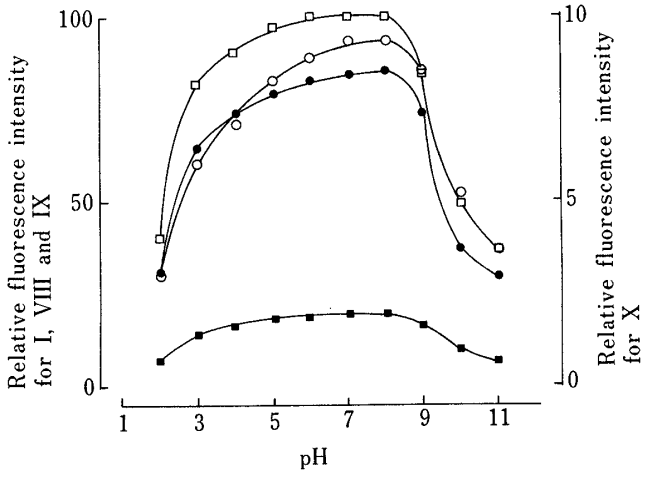
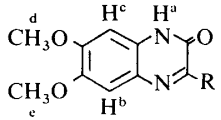


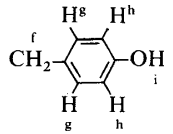
Fig. 1. Effect of pH on the Fluorescence Intensities of Compounds I, VIII, IX and X

Each compound (1×10^{-6} M) was dissolved in Britton–Robinson buffer of various pHs.
●—●, compound I; ○—○, compound VIII; □—□, compound IX; ■—■, compound X.

I, VIII, IX and X occurred at pH 6.0—8.0 (Fig. 1), and the other compounds also showed similar behavior. These compounds were stable in methanol and the phosphate buffer for at least 5 h. Their quantum yields were in the range of 0.42—0.56, except for compound X (Table

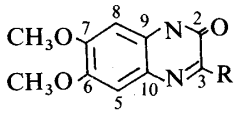
TABLE III. ^{1}H-NMR Data for 3-Substituted 6,7-Dimethoxy-2(1*H*)-quinoxalinones



Compound No.	R	Chemical shift (ppm)								
		H^a	H^b	H^c	H^d	H^e	H^f	H^g	H^h	H^i
I	CH_3	12.03 (1H, br s)	7.17 (1H, s)	6.76 (1H, s)	3.81 (3H, s)	3.84 (3H, s)	2.37 (3H, s)			
II	CH_2CH_3	12.04 (1H, br s)	7.19 (1H, s)	6.77 (1H, s)	3.83 (3H×2, s)		2.77 (2H, q, J=7)	1.20 (3H, t, J=7)		
III	$CH_2CH_2CH_3$	12.05 (1H, br s)	7.19 (1H, s)	6.77 (1H, s)	3.83 (3H×2, s)		2.73 (2H, t, J=7)	1.72 (2H, se, J=7)	0.95 (3H, t, J=7)	
IV	$CH(CH_3)_2$	12.06 (1H, br s)	7.20 (1H, s)	6.78 (1H, s)	3.84 (3H×2, s)		3.47 (1H, q, J=7)	1.21 (3H×2, d, J=7)		
V	$CH_2CH_2CH_2CH_3$	12.04 (1H, br s)	7.20 (1H, s)	6.77 (1H, s)	3.83 (3H×2, s)		2.76 (2H, t, J=7)	1.18—1.84 (2H×2, m)		0.93 (3H, t, J=6.5)
VI	$CH_2CH(CH_3)_2$	12.02 (1H, br s)	7.20 (1H, s)	6.77 (1H, s)	3.82 (3H×2, s)		2.63 (2H, d, J=7.5)	2.08—2.44 (1H, m)	0.93 (3H×2, d, J=6.5)	
VII	$CH(CH_3)CH_2CH_3$	12.07 (1H, br s)	7.21 (1H, s)	6.79 (1H, s)	3.85 (3H×2, s)		3.16—3.40 (1H, m)	1.33—2.00 (2H, m)	1.17 (3H, d, J=6.5)	0.86 (3H, t, J=7)
VIII	CH_2CH_2COOH	12.02 (1H, br s)	7.13 (1H, s)	6.77 (1H, s)	3.83 (3H×2, s)		2.98 (2H, t, J=6)	2.71 (2H, t, J=6)	3.37 (1H, br s)	
IX	$CH_2Ph(H5)$	12.15 (1H, br s)	7.19 (1H, s)	6.77 (1H, s)	3.81 (3H×2, s)		4.07 (2H, s)	7.19—7.34 (5H, m)		
X		12.11 (1H, br s)	7.21 (1H, s)	6.77 (1H, s)	3.83 (3H×2, s)		3.96 (2H, s)	7.12 (1H×2, d, J=8)	6.67 (1H×2, d, J=8)	9.14 (1H, s)

a) The signals of H^a disappeared on treatment with D_2O.

TABLE IV. ^{13}C-NMR Data for 3-Substituted 6,7-Dimethoxy-2(1*H*)-quinoxalinones



Compound No.	R	Chemical shift (ppm)													
		C-2	C-3	C-5[a]	C-6[b]	C-7[b]	C-8[a]	C-9[c]	C-10[c]	C-α	C-β	C-γ	C-δ	C-ε	OCH_3
I	CH_3 (α)	154.7	155.3	109.8	150.8	145.6	97.3	126.0	126.7	20.0					55.7 55.9
II	CH_2CH_3 (α, β)	154.4	158.9	109.9	150.8	145.6	97.3	126.0	126.6	25.6	10.6				55.7 55.9
III	$CH_2CH_2CH_3$ (α, β, γ)	154.8	158.0	109.9	150.9	145.7	97.3	126.0	126.6	34.5	19.5	13.8			55.7 55.9
IV	$CH(CH_3)_2$ (α, β)	154.0	162.0	109.9	150.9	145.7	97.3	125.8	126.5	29.6	20.0	20.0			55.7 55.9
V	$CH_2CH_2CH_2CH_3$ (α, β, γ, δ)	154.5	158.2	109.8	150.8	145.6	97.3	126.0	126.5	32.0	28.4	21.9	13.7		55.7 55.9
VI	$CH_2CH(CH_3)_2$ (α, β, γ, δ)	154.7	157.6	109.9	150.9	145.7	97.3	126.0	126.6	41.3	26.2	22.5	22.5		55.8 56.0
VII	$CH(CH_3)CH_2CH_3$ (α, β, γ, δ)	154.2	161.5	109.9	150.9	145.7	97.3	126.0	126.3	36.1	27.1	11.7	17.7		55.8 55.9
VIII	CH_2CH_2COOH (α, β, γ)	154.4	156.5	109.8	150.9	145.7	97.3	125.8	126.6	29.9	27.5	173.6			55.7 55.9
IX	CH_2-Ph (α, β, γ, δ, ε)	154.4	156.5	109.6	151.1	145.8	97.2	126.0	126.8	37.9	137.9	128.1[d]	128.9[d]	126.0	55.9 55.9
X	CH_2-Ph-OH (α, β, γ, δ, ε)	154.4	157.0	109.9	151.1	145.7	97.3	126.0	126.8	37.8	128.0	129.8[a]	115.0[a]	155.7	55.8 55.9

a) These assignments were confirmed by ^{1}H-selective hetero-spin decoupling. *b*—*d*) The assignments may be reversed.

TABLE V. Fluorescence Properties of 3-Substituted 6,7-Dimethoxy-2(1*H*)-quinoxalinones[a] in Methanol and Phosphate Buffer (0.04 M, pH 7.0)

Compound No.	Excitation maximum (nm)		Emission maximum (nm)		Relative fluorescence intensity[b]		Quantum yield	
	MeOH	Buffer	MeOH	Buffer	MeOH	Buffer	MeOH	Buffer
I	361	361	442	449	100	86	0.50	0.44
II	360	361	443	448	110	91	0.50	0.45
III	361	364	443	449	109	100	0.53	0.46
IV	360	362	442	448	102	96	0.47	0.44
V	362	363	443	449	113	95	0.51	0.46
VI	363	365	443	449	108	89	0.54	0.47
VII	362	364	443	449	106	92	0.47	0.42
VIII	359	361	444	449	105	95	0.50	0.47
IX	366	368	447	452	129	102	0.56	0.51
X	365	366	444	449	5.9	2.0	0.028	0.018

a) Concentration, 1×10^{-6} M. *b*) The fluorescence intensity of compound I in methanol was taken as 100.

V).

The fluorescence intensities were all approximately the same except for that of compound X. These intensities were approximately 100 times higher than those of the fluorescent compounds formed in the reaction of α-keto acids with *o*-phenylenediamine.[5] The small fluorescence intensity of compound X may be due to its low quantum yield.

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