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Synthesis and Chemiluminescence Properties of 6-(4-Methoxyphenyl)-2-methylimidazo[1,2-*a*]-pyrazin-3(7*H*)-one and 2-Methyl-6-(2-naphthyl)-imidazo[1,2-*a*]pyrazin-3(7*H*)-one

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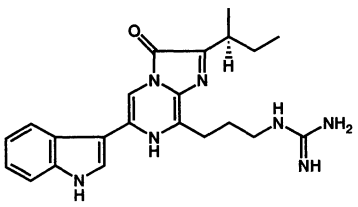
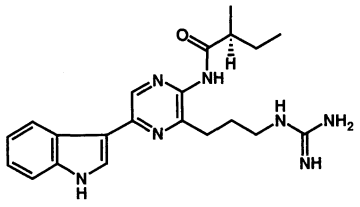
New *Cypridina* luciferin analogues, 6-(4-methoxyphenyl)-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (MCLA) and 2-methyl-6-(2-naphthyl)imidazo[1,2-*a*]pyrazin-3(7*H*)-one (NCLA), were prepared together with their oxyluciferin analogues, 2-acetamido-5-(4-methoxyphenyl)pyrazine (MCOLA) and 2-acetamido-5-(2-naphthyl)-pyrazine (NCOLA). Various chemiluminescence properties of these compounds were compared with those of known luciferin analogues, 2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (CLA) and 6-(3-indolyl)-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (ICLA). The light yields obtained from ICLA, MCLA, and NCLA were much higher than CLA in diethylene glycol dimethyl ether (diglyme), in which triplet oxygen was the oxidant. In aqueous solution, employing superoxide ion as the oxidant, NCLA emitted light weaker than CLA and MCLA. ICLA gave almost no light on account of no fluorescence intensity of the emitter (oxyluciferin analogue ICOLA) in aqueous solution. For applications to the detection of active oxygen species produced during enzymatic reactions, MCLA is superior to conventionally used CLA because of its higher chemiluminescence quantum yield and its emission maximum in visible region.

Cypridina bioluminescence is produced in aqueous solution by oxidation of *Cypridina* luciferin (the substrate) with molecular oxygen in the presence of *Cypridina* luciferase (the enzyme).[1)]

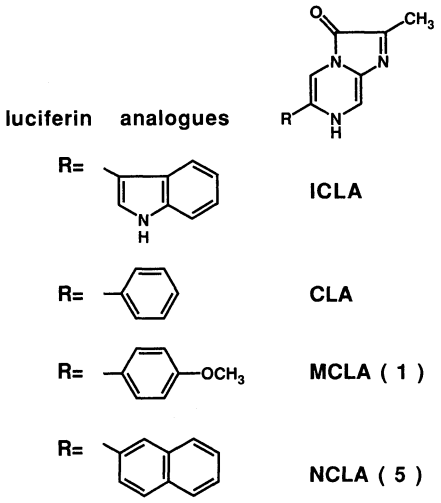
Cypridina luciferin is one of the best chemiluminescent substances.[1)] In the presence of triplet oxygen (air), light emission occurs in aprotic polar solvents such as diethylene glycol dimethyl ether (diglyme) containing a trace of acetate buffer (pH 5.6). In aqueous solutions, however, no light emission occurs even if active oxygen such as superoxide or singlet oxygen present. We found that a *Cypridina* luciferin analogue, 2-methyl-6-phenyl-imidazo[1,2-*a*]pyrazin-3(7*H*)-one (CLA), gives light in aqueous solution in the presence of active oxygen as well as in aprotic polar solvent with triplet oxygen. This may be because *C.* oxyluciferin, the emitter produced by the oxidation of *C.* luciferin, shows no fluorescence in aqueous solutions, whereas the *C.* oxyluciferin analogue

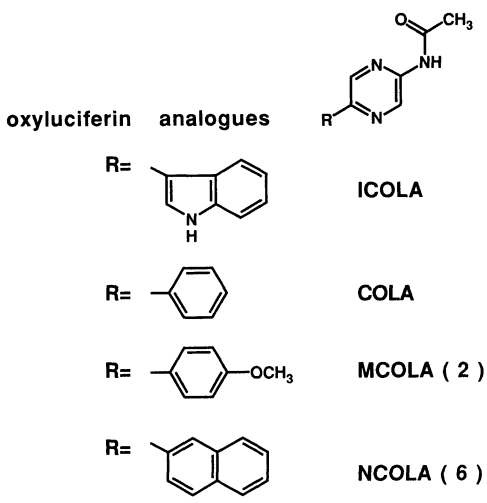


***Cypridina* luciferin**



***Cypridina* oxyluciferin**





(COLA) produced from CLA shows strong fluorescence in aqueous solutions.[2]

CLA reacts with superoxide anion (or hydroperoxide radical) or singlet oxygen to give chemiluminescence in aqueous solutions. This reaction has been used for the quantitative analysis of such active oxygens.[3] To find more effective reagents we synthesized two other *Cypridina* luciferin analogues, 6-(4-methoxyphenyl)-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (MCLA) (**1**) and 2-methyl-6-(2-naphthyl)imidazo[1,2-*a*]pyrazin-3(7*H*)-one (NCLA) (**5**), and their corresponding oxyluciferin analogues, 2-acetamido-5-(4-methoxyphenyl)-pyrazine (MCOLA) (**2**) and 2-acetamido-5-(2-naphthyl)pyrazine (NCOLA) (**6**), according to the similar methods as previously described.[4]

2-Naphthylglyoxal aldoxime obtained from 2-acetylnaphthalene was condensed with aminoacetonitrile in the presence of titanium tetrachloride to give 2-amino-5-(2-naphthyl)pyrazine 1-oxide (**3**), which was reduced with Raney nickel to the naphthylpyrazine **4**. The naphthylpyrazine **4** was condensed with methylglyoxal to give NCLA (**5**). MCLA (**1**) was obtained similarly from 2-amino-5-(4-methoxyphenyl)pyrazine.

Figure 1 shows the electronic spectra of **1** and **5**, as well as CLA and ICLA.[5] Figure 2 shows the electronic spectra of the corresponding oxyluciferin analogues (COLA, MCOLA, NCLOA, and ICOLA) in methanol. Fluorescence spectra of these compounds in diglyme and in water are shown in Figs. 3 and 4, respectively. The fluorescence intensity of MCOLA and NCOLA is nearly three and two times, respectively, stronger than that of COLA in diglyme, whereas in water NCOLA shows much weaker fluorescence than those of COLA and MCOLA (*C.* oxyluciferin and ICOLA show no fluorescence in aqueous solutions).[2]

These results are consistent with the observation (Table 1) that much higher light yields were obtained from MCLA and NCLA in diglyme than that from CLA, whereas in aqueous solution (superoxide ion was the oxidant) NCLA emitted light weaker than CLA and MCLA. As reported previously,[1,3] ICLA shows very strong chemiluminescence in diglyme, but almost no light in aqueous solution, because ICOLA shows no fluorescence in aqueous solutions.

It should be noted that the chemiluminescence spectra of MCLA and ICLA in diglyme (Fig. 5) are nearly

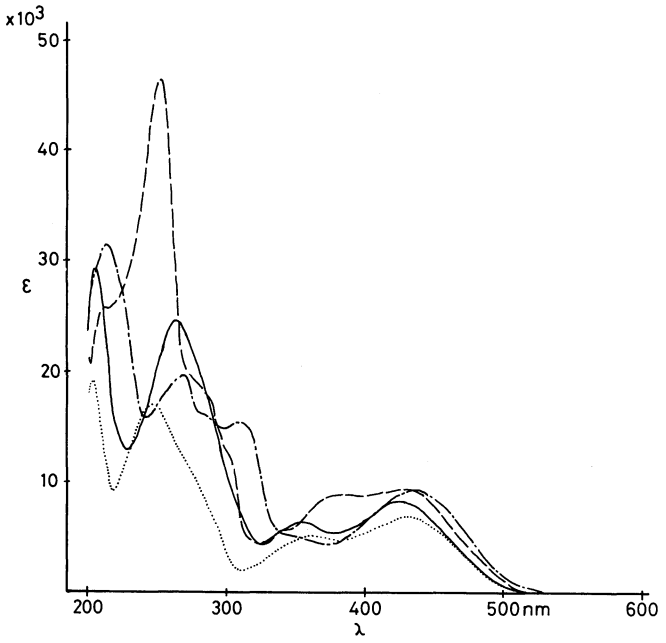


Fig. 1. Electronic spectra of *C.* luciferin analogues (HCl salt) in methanol. ····· CLA; —— MCLA; --- NCLA; —·— ICLA.

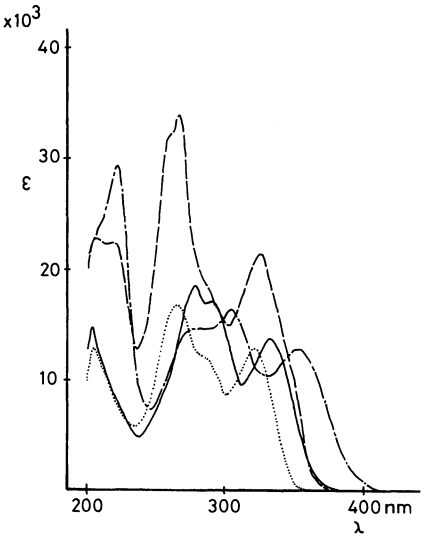


Fig. 2. Electronic spectra of *C.* oxyluciferin analogues in methanol. ····· COLA; —— MCOLA; --- NCOLA; —·— ICOLA.

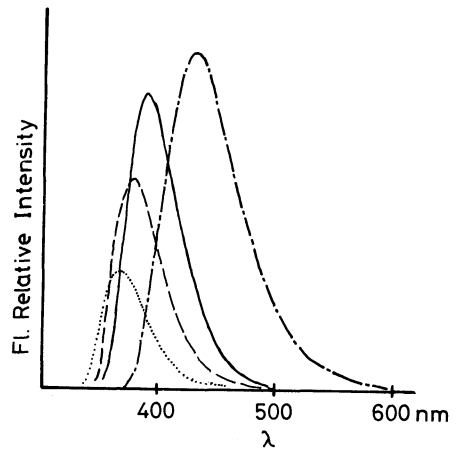


Fig. 3. Fluorescence spectra of *C.* oxyluciferin analogues in diglyme (at 25°C). ····· COLA; —— MCOLA; --- NCOLA; —·— ICOLA. Conditions: concn, 1.26—2.77×10^{-6} mol dm^{-3}. Relative intensity was corrected to the same concn; excitation, at each longest UV maximum; additive, none.

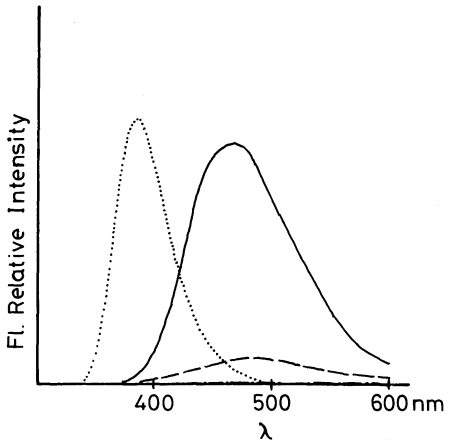


Fig. 4. Fluorescence spectra of *C.* oxyluciferin analogues in water (at 25°C). ····· COLA; —— MCOLA; --- NCOLA; ——— ICOLA. Conditions: concn, 1.26—2.77×10^{-6} mol dm^{-3}. Relative intensity was corrected to the same concn; excitation, at each longest UV maximum; additive, none.

Table 1. Relative Light Yields of Chemiluminescence (at 25°C)

Compound	CLA	MCLA	NCLA	ICLA
Light yield in diglyme[a)]	430	3400	1900	10000
Light yield in X-XOD[b)]	110	140	30	2

a) To a mixed solution of diglyme (3 ml) and 0.1 mol dm^{-3} acetate buffer (pH 5.6, 75 μl) was added each 1×10^{-3} mol dm^{-3} luciferin analogue solution in MeOH (5 μl). b) To 0.1 mol dm^{-3} acetate buffer (pH 5.6, 3.0 ml), containing 1×10^{-4} mol dm^{-3} EDTA 2Na and 4×10^{-4} mol dm^{-3} hypoxanthine, was added XOD (5μl, 0.05 units) and incubated for 4 min. To this X-XOD system was added each 1×10^{-3} mol dm^{-3} luciferin analogue solution in MeOH (5 μl).

identical with the fluorescence spectra of the corresponding oxyluciferin analogues, MCOLA and ICOLA, respectively, indicating that the emitter is a neutral molecule in each case. In the case of CLA and NCLA, however, the emitting species is mainly the amido anion of COLA and NCOLA as evident from the two peaks in their chemiluminescence spectra; a peak at shorter wavelength (ca. 380 nm) corresponds to the neutral molecule, and another peak at longer wavelength (ca. 460 nm) to the amido anion.[6)]

Chemiluminescence spectra of CLA and MCLA in aqueous solution (pH 5.6) containing the xanthine–xanthine oxidase system (the X-XOD system),[3)] in which superoxide ion is produced, are shown in Fig. 6. These compounds chemiluminesce in physiological conditions (pH 5—7) with superoxide ion or singlet oxygen. Wide applications of them to the detection of such active oxygen species produced by enzymatic reactions would be expected. For these applications, MCLA may be

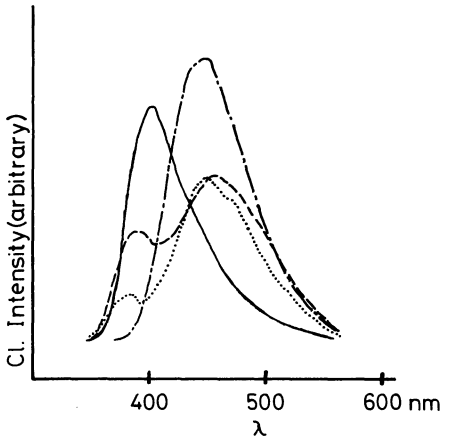


Fig. 5. Chemiluminescence spectra in diglyme (containing acetate buffer, at 25°C). ····· CLA; —— MCLA; --- NCLA; ——— ICLA. Conditions: same as described on Table 1 except 10 μl of CLA and NCLA solutions in MeOH were used instead of 5 μl.

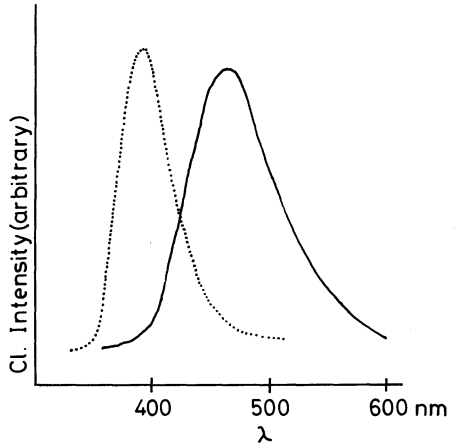


Fig. 6. Chemiluminescence spectra in the presence of X-XOD system (at 25°C). ····· CLA; —— MCLA. Conditions: same as described on Table 1 except 2×10^{-4} mol dm^{-3} of X, 30 μl (0.3 units) of XOD, and 10 μl of 5×10^{-3} mol dm^{-3} CLA and MCLA solutions in MeOH were used instead of hypoxanthine and corresponding materials.

superior to CLA since the chemiluminescence quantum yield of MCLA is higher than that of CLA, and the emission maximum of MCLA (460 nm) is in the visible region; the emission in the visible region is better because of the possible quenching by impurities in the ultraviolet region. Some applications of MCLA to analysis of superoxide ion have been reported, and it has been shown that maximum light intensity from MCLA is 4.6 times stronger than that from CLA under the reported conditions.[7)]

Experimental

All melting points were measured on a Mitamura Riken mp apparatus and uncorrected. ^{1}H NMR spectra were recorded on a JEOL JNM-FX200 spectrometer. Chemical shifts (δ)

were given in ppm from internal TMS and coupling constants (J) in Hz. UV spectra were measured on a JASCO UVIDEC-660 spectrometer. Mass spectra were measured on a JEOL JMS-D100 instrument. Fluorescence spectra were recorded on a Hitachi 850 spectrofluorometer and corrected for lamp intensity and photomultiplier (PMT) response characteristics. Chemiluminescence rates and light yields were obtained using an automatic recording PMT (Hamamatsu R105H)-amplifier system previously described,[8] or a Hamamatsu C-1230 photon counter controlled by an NEC PC-9801VX personal computer, with a homemade shielding measurement box containing a PMT (Hamamatsu R1527P). These chemiluminescence data were uncorrected for PMT response and transmittance of Pyrex glass vial and extinction filter (Toshiba TND-0.1). Chemiluminescence spectra were recorded on a JASCO FP-770 or a JASCO FP-777 spectrofluorometer and uncorrected. Diethylene glycol dimethyl ether (diglyme) was distilled from sodium or calcium hydride and stored at −20 °C. Xanthine oxidase (XOD) was purchased from Sigma Chemical Co. (X 1875) and used without desalting. The other solvents and chemicals were reagent grade and used without purification.

6-(4-Methoxyphenyl)-2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one Hydrochloride (MCLA) (1): To a mixture of 2-amino-5-(4-methoxyphenyl)pyrazine (100 mg) and methylglyoxal (40% aqueous soln, 0.16 ml) in methanol (4.1 ml) was added concd hydrochloric acid (0.10 ml). The resulting orange solution was sealed in a flask with a rubber septum under a nitrogen atmosphere and heated at 70 °C (bath temp) for 1.5 h with stirring. After the solution was evaporated to dryness under vacuum, the residue was chromatographed on a silica-gel column (10 g; solvent: 2-propanol). The product was crystallized from benzene–methanol to give **1** as pale yellow crystalline powder (89 mg, 61%); mp 197—201 °C (decomp); ^{1}H NMR (DMSO-d_6+D_2O) δ=2.36 (3H, s, CH_3), 3.82 (3H, s, CH_3O), 7.09 and 7.71 (2H and 2H, $A_2'B_2'$, J=9 Hz), 7.70 (1H, s), 8.05 (1H, s); MS (EI) m/z 255 (M^+), 240, 226; UV (MeOH) λ_{max} (ε) 430 (8260), 357 (5460), 261 (20800), 203 (20900) nm; (MeOH–HCl) 348 (5080), 278 (21700), 208 (18600) nm; (MeOH–NaOH) 409 (6000), 348 (5530), 265 (22900), 207 (20900) nm. Calcd for $C_{14}H_{14}O_2N_3Cl$: C, 57.64; H, 4.84; N, 14.40%. Found: C, 57.62; H, 4.70; N, 14.39%.

2-Acetamido-5(4-methoxyphenyl)pyrazine (MCOLA) (2): A mixture of 2-amino-5-(4-methoxyphenyl)pyrazine (100 mg), pyridine (5 ml), and acetic anhydride (1.2 ml) was stirred at room temp overnight and then at 50 °C for 4 h. The reaction mixture was evaporated under vacuum and the residue was crystallized from ethyl acetate to give **2** as pale brown needles (98 mg, 81%), mp 183—185 °C; MS (EI) m/z 243 (M^+), 201; UV (MeOH) λ_{max} (ε) 332 (14200), 290 (17700), 278 (18600), 202 (12600) nm; ^{1}H NMR ($CDCl_3$) δ=2.27 (3H, s), 3.87 (3H, s), 7.01 and 7.93 (2H and 2H, $A_2'X_2'$, J=9 Hz), 7.89 (1H, broad s, NH), 8.60 (1H, d, J=1.5 Hz), 9.51 (1H, broad s). Calcd for $C_{13}H_{13}O_2N_3$: C, 64.20; H, 5.35; N, 17.28%. Found: C, 64.11; H, 5.37; N, 17.49%.

2-Amino-5-(2-naphthyl)pyrazine 1-Oxide (3): To a sodium ethoxide solution obtained from sodium metal (1.92 g) and absolute ethanol (50 ml) was added with stirring 2-acetylnaphthalene (11.35 g). After the solid had dissolved, the resulting solution was treated with isopentyl nitrite (10 ml) by dropwise addition and then allowed to stand for five days with stirring at room temp in the dark. The mixture was filtered, the solid was washed with absolute ethanol and dried over P_2O_5 under vacuum to give 2-naphthylglyoxal aldoxime as a reddish brown powder (9.8 g, 86%), which was used for the next step without further purification.

To a solution of the aldoxime (1.26 g) and aminoacetonitrile hydrogensulfate (0.815 g) in pyridine (20 ml) was added $TiCl_4$ (0.63 ml) dropwise under a nitrogen atmosphere with cooling in an ice-salt bath. The mixture was heated at 80 °C for 1.5 h with stirring and then poured into a mixture of saturated aq sodium hydrogencarbonate (200 ml) and ethyl acetate (50 ml). The mixture was filtered under reduced pressure and the cake was washed with ethyl acetate. The filtrate was acidified with concd hydrochloric acid to pH 3—4 and extracted with ethyl acetate (50 ml each) four times. The combined extracts were dried over Na_2SO_4 and evaporated under vacuum. The residual dark brown solid was chromatographed on a silica-gel column with 5% methanol–CH_2Cl_2 to give two fractions. The latter fraction containing mainly the *N*-oxide **3** was further purified by silica-gel column chromatography using ethyl acetate as the eluting solvent to give the *N*-oxide (230 mg), which was crystallized from ethyl acetate to yield **3** as pale brown crystalline powder (141 mg, 11%), mp 245.5—246 °C; UV (MeOH) λ_{max} (ε) 357 (5120), 306 (20900), 261 (37500), 212 (24000) nm; ^{1}H NMR (CD_3OD) δ=7.52 (2H, m), 7.81—7.98 (3H, m), 8.01 (1H, dd, J=9, 2 Hz), 8.37 (1H, d, J=0.5 Hz), 8.39 (1H, m), 8.67 (1H, d, J=0.5 Hz). Calcd for $C_{14}H_{11}ON_3$: C, 70.87: H, 4.64; N, 17.71%. Found: C, 70.88; H, 4.90; N, 17.44%.

2-Amino-5-(2-naphthyl)pyrazine (4): To a solution of the *N*-oxide **3** (50 mg) in a mixture of ethanol (4 ml) and ethyl acetate (4 ml) was added Raney Ni (W-2, 0.3 g) and the mixture was stirred under a hydrogen atmosphere at room temp for 3.2 h. The product was crystallized from benzene to give **4** as pale brown plates (31 mg, 67%), mp 154—156 °C; UV (MeOH) λ_{max} (ε) 331 (11500), 301 (19200), 293 (19100), 270 (30500), 218 (31200) nm; (MeOH–HCl) 355 (6780), 302 (21700), 292 (21900), 267 (35800), 217 (30200) nm; ^{1}H NMR (CD_3OD) δ=7.48 (2H, m), 7.79—7.96 (3H, m), 7.99 (1H, dd, J=8.8, 1.7 Hz), 8.06 (1H, d, J=1.7 Hz), 8.30 (1H, m), 8.52 (1H, d, J=1.7 Hz). Calcd for $C_{14}H_{11}N_3$: C, 76.00; H, 5.01; N, 18.99%. Found: C, 76.05; H, 5.12; N, 18.86%.

2-Methyl-6-(2-naphthyl)imidazo[1,2-*a*]pyrazin-3-(7*H*)-one Hydrochloride (NCLA) (5): A mixture of the naphthylpyrazine **4** (145 mg), 40% aq methylglyoxal (0.40 ml), concd hydrochloric acid (0.20 ml), and methanol (9 ml) in a flask sealed with a rubber septum was heated at 90 °C (bath temp) with stirring for 7.5 h and then evaporated under vacuum. The yellow residue was heated in 2-propanol (20 ml) and the mixture was filtered and washed with 2-propanol to give a solid (125 mg, 62%), which was crystallized from methanol to yield **5** as pale brown crystalline powder. Mp 253—256 °C; UV (MeOH) λ_{max} (ε) 425 (9070), 385 (8780), 250 (46500), 211 (26800) nm; (MeOH–HCl) 286 (20200), 251 (45600), 210 (27800) nm; (MeOH–NaOH) 371 (9020), 338 (6220), 288 (22900), 254 (47000), 214 (30900) nm; ^{1}H NMR (CD_3OD) δ=2.54 (3H, s), 7.56 (2H, m), 7.81—8.11 (4H, m), 8.50 (1H, m), 8.68 (1H, d, J=1.1 Hz), 8.99 (1H, d, J=1.1 Hz). Calcd for $C_{17}H_{14}ON_3Cl$: C, 65.49; H, 4.53; N, 13.48%. Found: C, 65.49; H, 4.59; N, 13.44%.

2-Acetamido-5-(2-naphthyl)pyrazine (NCOLA) (6): A mixture of 2-amino-5-naphthylpyrazine (**4**) (100 mg), pyridine (6 ml), and acetic anhydride (0.75 ml) was heated at 50 °C for

1 d, and then evaporated to dryness under vacuum. The residue was crystallized from benzene–methanol to give **6** as pale yellow needles (98 mg, 82%), mp 203—204°C; UV (MeOH) λ_{max} (ε) 325 (21500), 266 (34100), 219 (22500), 206 (22800) nm; ^{1}H NMR (CD_3OD) δ=2.23 (3H, s), 7.53 (2H, m), 7.87—8.00 (3H, m), 8.14 (1H, dd, J=8.8, 1.7 Hz), 8.51 (1H, broad d, J=1.2 Hz), 8.97 (1H, d, J=1.5 Hz), 9.44 (1H, d, J=1.5 Hz). Calcd for $C_{16}H_{13}ON_3$: C, 72.99; H, 4.98; N, 15.96%. Found: C, 72.89; H, 4.99; N, 16.01%.

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