SYNTHESIS OF 6-(BROMOACETYL)AMINO-2,3-DIMORPHOLINO-QUINOXALINE AND APPLICATION TO A NEW FLUORESCENCE DERIVATIZATION REAGENT OF FATTY ACIDS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

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Abstract — 6-Amino-2,3-dimorpholinoquinoxaline (**3**) was allowed to react with bromoacetic acid in the presence of DCC to give 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline (**4**). Five kinds of saturated fatty acids (C_{10}, C_{12}, C_{14}, C_{16}, and C_{18}) were subjected to the derivatization with compound (**4**) within 50 min to afford the corresponding fluorescent products (**5a-e**). All the peaks of the derivatized products clearly separated within 15 min. The detection limit of decanoic acid (C_{10}) was estimated to be 10 fmol/10 μL injection volume (S/N=4).

The high-sensitive determination of biological substances such as carboxylic acids, amines, amino acids, alcohols, and ketones is indispensable for diagnosis of sickness, elucidation of physiological function, and the chemotheraputic treatment.[1] Recently, high sensitive and selective HPLC method with a fluorescence detector has received much attention for the detection of bioactive substances and related compounds using fluorescence derivatization reagents.[2-4] In general, the following physical and photometric characteristics are required for the fluorescence derivatization reagent; 1) it emits intense fluorescence, 2) excitation and emission bands appear at longer wavelength region, and a difference in wavelength between two bands is wide, 3) the derivatization rapidly and quantitatively proceeds under mild conditions, 4) the fluorescence property is not influenced by analytical conditions, 5) the molecular size of the labelling reagent is small, and 6) the labelling reagent and derivatized product are stable at room temperature. A variety of fluorescence derivatization reagents including bromomethyl,[5-10]

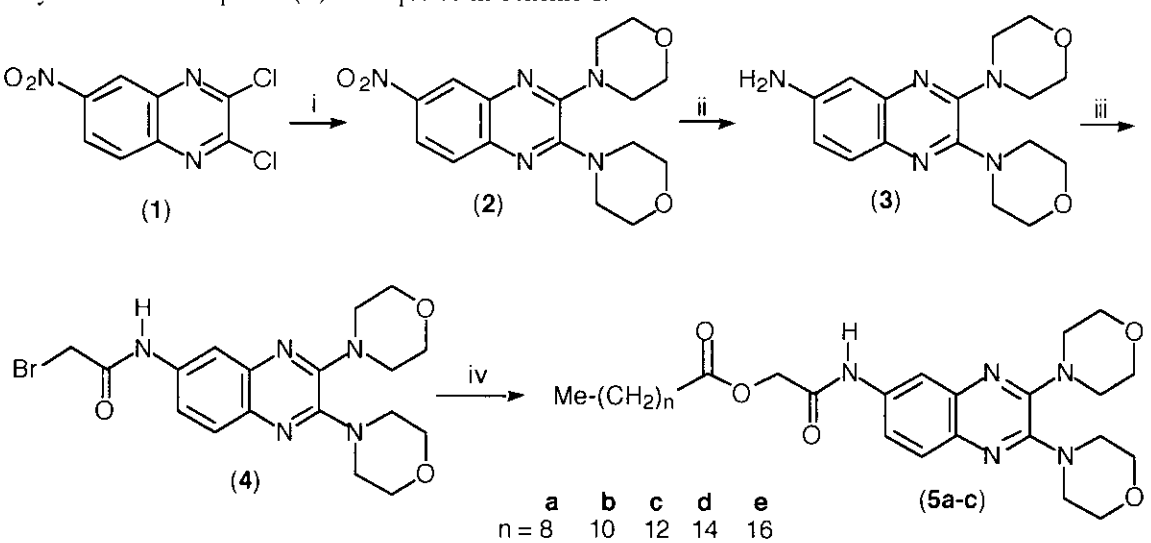
bromoacetyl,[11-14] diazomethyl,[15,16] amino,[17,18] hydorazino,[19] trifluoromethanesulfonyl[20,21] groups as reacting species have been developed for high sensitive determination of carboxylic acids. However, some of these reagents have disadvantages in terms of stability and sensitivity, and thus design and synthesis of new fluorescence derivatization reagents are still needed.

Recently, in our laboratory, 2,3-disubstituted 6-aminoquinoxaline has been demonstrated to be a new high-sensitive fluorescence derivatization reagent for carboxylic acids.[22] However, this compound required long derivatization time owing to low reactivity of the amino group at C-6 position of the quinoxaline ring. For the purpose of improvement of this disadvantage, we would like to describe here a synthesis of 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline and its application to a fluorescence derivatization reagent for fatty acids.

RESULTS AND DISCUSSION

Synthesis and Derivatization

Synthesis of 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline (**4**) and the fluorescence derivatization of fatty acids with compound (**4**) are depicted in Scheme 1.



Scheme 1. Reagents and conditions: i) morpholine in DMF, rt; ii) H_2/10% Pd-C in MeOH; iii) $BrCH_2CO_2H$/DCC in DMF, rt; iv) $Me(CH_2)_nCO_2H$/18-crown-6/$KHCO_3$ in MeCN

6-Amino-2,3-dimorpholinoquinoxaline (**3**) was selected as the substrate for the bromoacetylation, because it showed the most excellent fluorescence characteristics among four kinds of 2,3-disubstituted 6-

aminoquinoxalines[22] reported previously. 2,3-Dichloro-6-nitroquinoxaline (**1**)[23] was treated with an excess of morpholine in DMF to give 2,3-dimorpholino-6-nitroquinoxaline (**2**). Compound (**2**) was subjected to the hydrogenation in the presence of 10% Pd-C to give 6-amino-2,3-dimorpholinoquinoxaline (**3**). The *N*-bromoacetylation of compound (**3**) was carried out using three methods, and the results are summarized in Table 1. The first two methods (A and B) afforded 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline (**4**) in moderate yields, but a number of by-product spots were detected on TLC. On the other hand, method C gave the product (**4**) in almost quantitative yield.

Table 1. The *N*-Bromoacetylation of 6-Amino-2,3-dimorpholinoquinoxaline (**3**)

Method	Reagent	Conditions	Yield (**4**, %)
A	$BrCH_2COCl$	Et_3N in CH_2Cl_2 at 0 °C for 15 min	55
B	$BrCH_2COBr$	3M NaOH in dioxane at 5 °C for 30 min	66
C	$BrCH_2CO_2H$	DCC in DMF at rt for 4 h	97

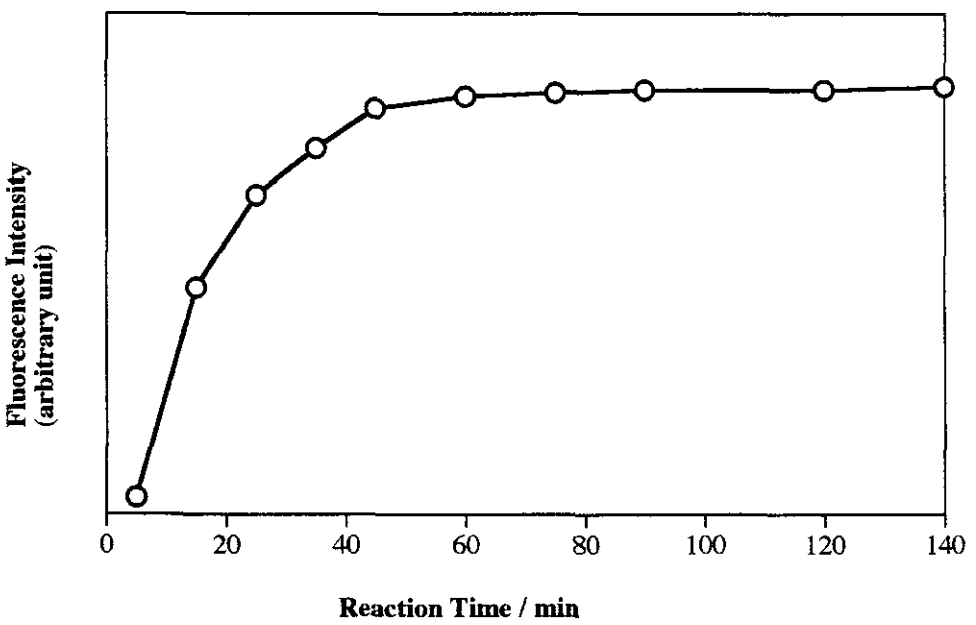


Figure 1. The Reaction Profile for Derivatization of Decanoic Acid with Compound (**4**)
[decanoic acid]=[**4**]=[18-crown-6]=7 mM, [$KHCO_3$]=0.21 M; Ex. 368 and Em. 441 nm

The derivatization time is one of the most important factors for the fluorescence derivatization reagent. The derivatization of decanoic acid with an equimolar amount of compound (**4**) was carried out in the presence

of an equimolar amount of 18-crown-6 and 30-fold excess of $KHCO_3$ in MeCN at room temperature, and the reaction profile is shown in Figure 1. The derivatization smoothly proceeded within 50 min, and the isolated yield of the derivatized product (**5a**) was actually 97%. Similarly, other four kinds of fatty acids were derivatized in the same fashion to give the products (**5b-e**). It is noteworthy that the derivatized products (**5a-e**) emit a intense fluorescence.

Characteristics of UV-Vsible and Fluorescence Spectra of the Derivatized Products

Spectral data of UV-visible and fluorescence of the derivatized product, 6-(*O*-decanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (**5a**) both in MeCN and in MeOH are listed in Table 2. Compound (**5a**) showed the absorption maximum at around 370 nm with ε about 16000.

Table 2. Absorption and Fluorescence Spectral Data of the Derivatized Product (**5a**)

Solvent	Absorption[a]		Fluorescence[b]		
	λ_{max}/nm	ε	Ex./nm	Fλ_{max}/nm	RFI[c]
MeCN	368	15950	368	441	1.0
MeOH	367	16150	364	443	0.1

a) [**5a**] = 1 x 10^{-4} M. b) [**5a**] = 1 x 10^{-6} M. c) Relative fluorescence intensity (RFI) of **5a** in MeCN is arbitrarily taken as 1.0.

Table 3. The Influence of Water Concentration on the Relative Fluorescence Intensity of the Derivatized Product (**5a**) in Aqueous MeCN

Water Conc.	Fluorescence			Water Conc.	Fluorescence		
%	Ex./nm	Fλ_{max}/nm	RFI[c]	%	Ex./nm	Fλ_{max}/nm	RFI[c]
0	368	441	1.00	50	373	447	0.05
10	370	445	0.11	60	373	447	0.04
20	368	445	0.09	70	372	449	0.03
30	373	446	0.07	80	371	449	0.02
40	373	446	0.06	90	367	449	0.01

Compound (**5a**) showed an emission band at around 440 nm, but the relative fluorescence intensity in MeOH was one-tenth compared to that in MeCN. Further, the influence of water concentration in aqueous MeCN on the fluorescence intensity of the derivatized product (**5a**) was examined in the range of 0 to 90 %, and the results are summarized in Table 3. Even in the presence of small amount of water the relative fluorescence intensity dramatically decreased.

HPLC Diagram of Five Kinds of Fluorescent Derivatized Products

The detection limit for the derivatized product (**5a**) was estimated by employing a reversed-phase HPLC equipped with a fluorescence detector under conditions shown in the EXPERIMENTAL section. A linear relationship between the peak area and the amount of compound (**5a**) was observed in the range of 10 fmol to 1 nmol/10 μL injection volume (a correlation coefficient: 0.996), and thus the detection limit was estimated to be 10 fmol/10 μL (S/N=4). The simultaneous separation of the derivatized products (**5a-e**) of five kinds of fatty acids was attempted under the same HPLC conditions. A typical chromatogram of the derivatized products (**5a-e**) is shown in Figure 2. All the peaks were completely separated within 15 min. This result suggests that 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline is applicable to a fluorescence derivatization reagent for saturated fatty acids.

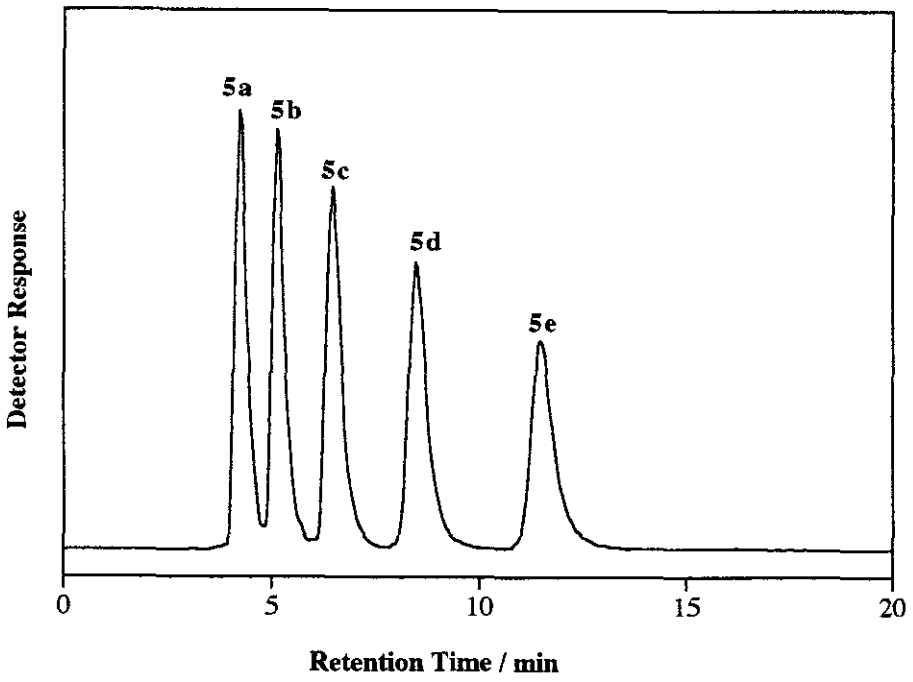


Figure 2. Chromatogram of a Mixture of the Derivatized Products; **5a**: Decanoic Acid; **5b**: Dodecanoic Acid; **5c**: Tetradecanoic Acid; **5d**: Hexadecanoic Acid; **5e**: Octadecanoic Acid.

EXPERIMENTAL

Melting points were recorded on a Mel-Temp apparatus in open capillaries and are uncorrected. IR spectra were recorded on a JASCO FT/IR-230 infrared spectrophotometer. UV-Visible and fluorescence spectra were recorded on JASCO Ubest V-550 and JASCO FP-777 fluorescence spectrophotometers, respectively.

^{1}H NMR spectra were recorded on JEOL GX-270 and JNM-LA400D spectrometers and are reported in ppm (δ) downfield from internal Me_4Si. Thin layer chromatographic (TLC) analyses were performed on silica gel 60F-254 with a 0.2 mm layer thickness. Column chromatography was carried out with Merck Kieselgel 60 (230-400 mesh). HPLC was carried out with a JASCO 880-PU, a 875-UV and a 821-FP equipped with a JASCO 807-IT integrator by using a column packed with a Finepak SIL $C_{18}S$ (flow rate: 1.0 mL/min; mobile phase: MeCN; attenuation: 1 mV; response: fast; gain: x10; λ_{ex}: 368 nm; λ_{em}: 441 nm). Combustion analyses were performed on a Yanaco MT-3 CHN CORDER and a Perkin Elmer Series II CHNO/S Analyser 2400.

2,3-Dimorpholino-6-nitroquinoxaline (2)

Morpholine (5 mL) was added dropwise to a solution of 2,3-dichloro-6-nitroquinoxaline (**1**) (0.25 g, 1 mmol) in DMF (5 mL) with stirring for 5 min. The resulting precipitate was collected by suction filtration, washed with H_2O (200 mL) and dried *in vacuo* to give the product (**2**); mp 179-180 °C (AcOEt-hexane); yield 0.35 g (99%); IR (KBr) 1512 and 1345 cm^{-1}; ^{1}H NMR ($CDCl_3$, 270 MHz) δ=3.57-3.63 (4H, m), 3.65-3.71 (4H, m), 3.86 (8H, m), 7.74 (1H, d, *J*=9.3 Hz), 8.23 (1H, dd, *J*=2.3 and 9.3 Hz), 8.60 (1H, d, *J*=2.3 Hz). *Anal.* Calcd for $C_{16}H_{19}N_5O_4$: C, 55.65; H, 5.55; N, 20.28. Found: C, 55.79; H, 5.66; N, 20.22.

6-Amino-2,3-dimorpholinoquinoxaline (3)

A solution of 2,3-dimorpholino-6-nitroquinoxaline (**2**) (0.7 g, 2.03 mmol) in distilled MeOH (300 mL) was hydrogenated for 2 h in the presence of 10% Pd-C (0.2 g) under hydrogen atmosphere. After removal of the catalyst, the solvent was evaporated to give the product (**3**) as grayish yellow powders; mp 229-231 °C; yield 0.59 g (93%); IR (KBr): 3448 and 1109 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ=3.43-3.50 (4H, m), 3.57-3.64 (4H, m), 3.83-3.87 (8H, m), 6.89 (1H, dd, *J*=8.5 and 2.4 Hz), 6.93 (1H, d, *J*=2.4 Hz), 7.55 (1H, d, *J*=8.5 Hz). *Anal.* Calcd for $C_{16}H_{21}N_5O_2$: C, 60.94; H, 6.71; N, 22.21. Found: C, 60.92; H, 6.68; N, 21.96.

6-(Bromoacetyl)amino-2,3-dimorpholinoquinoxaline (4)

Method A

To a solution of 6-amino-2,3-dimorpholinoquinoxaline (**3**) (27 mg, 8.5 x10^{-5} mol) and Et_3N (13 mg, 1.3x10^{-4} mol) in dry CH_2Cl_2 (10 mL) was added bromoacetyl chloride (20 mg, 1.3 x10^{-4} mol), and then the reaction mixture was stirred at 0 °C for 15 min. The reaction mixture was poured onto ice water.

The organic layer was washed with H_2O (20 mLx2) and saturated NaCl (20 mL), and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was chromatographed on silica gel with $CHCl_3$-acetone-EtOH (100:5:1) mixture to give the product (**4**) as pale brown powders; mp 183-185 °C; yield 20 mg (55%); IR (KBr) 3448, 1671 and 1117 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ=3.52-3.64 (8H, m), 3.81-3.90 (8H, m), 4.07 (2H, s), 7.55 (1H, dd, *J*=2.2 and 8.9 Hz), 7.70 (1H, d, *J*=8.9 Hz), 8.00 (1H, d, *J*=2.2 Hz), 8.32 (1H, br s). *Anal.* Calcd for $C_{18}H_{22}N_5O_3Br \cdot 1.3H_2O$: C, 47.03; H, 5.10. Found: C, 47.18; H, 4.92.

Method B

To a solution of 6-amino-2,3-dimorpholinoquinoxaline (**3**) (100 mg, 0.32 mmol) in dry dioxane (10 mL) was added bromoacetyl bromide (83 mg, 0.41 mmol) and 3M NaOH (0.14 mL, 0.41 mmol), and then the reaction mixture was stirred at 5 °C for 30 min. The reaction mixture was diluted with H_2O (30 mL) and the mixture was extracted with $CHCl_3$ (100 mL). The organic layer was washed with H_2O (30 mLx3), and dried over anhydrous Na_2SO_4. After removal of the solvent, the same work-up gave the product (**4**) (92 mg, 66%).

Method C

A solution of bromoacetic acid (0.88 g, 6.3 mmol) and DCC (1.3 g, 6.3 mmol) in dry DMF (35 mL) was stirred for 1 min at rt. To the mixture was added dropwise a solution of 6-amino-2,3-dimorpholino-quinoxaline (**3**) (0.2 g, 0.63 mmol) in dry DMF (5 mL), and then the reaction mixture was stirred for 4 h. After removal of the solvent, AcOEt (100 mL) was added to the residue, the precipitated *N,N'*-dicyclohexylurea was filtered off, and then the filtrate was evaporated off. The crude product was purified by column chromatography on silica gel with $CHCl_3$-acetone-EtOH (100:5:1) mixture and subsequent recrystallization from $CHCl_3$-hexane mixture to give the pure product (**4**) (0.27 g, 97%).

General Procedure for Derivatization of Fatty Acids with the Reagent (4). A Typical Example; 6-(*O*-Decanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (5a)

To a solution of 6-(bromoacetyl)amino-2,3-dimorpholinoquinoxaline (**4**) (30 mg, 6.9×10^{-5} mol), decanoic acid (12 mg, 7×10^{-5} mol), and 18-crown-6 (18 mg, 7×10^{-5} mol) in dry MeCN (10 mL) was added $KHCO_3$ (210 mg, 2.1×10^{-3} mol), and then the reaction mixture was stirred for 50 min at rt. After evaporation of the solvent, the residue was purified by column chromatography on silica gel with $CHCl_3$-acetone-EtOH (100:10:2) mixture to give the product (**5a**) as pale yellow powders; mp 116-117 °C; yield 36 mg (98%); IR (KBr): 3299 (νN-H), 1748 (νC=O), 1673 (νC=O), and 1117 cm^{-1} (νC-O-C); ^1H

NMR ($CDCl_3$, 270 MHz) δ=0.87 (3H, t, *J*=6.6 Hz, $-CH_3$), 1.23-1.41 (12H, m, $-(CH_2)_6-$), 1.66-1.77 (2H, m, $-CH_2CH_2COO-$), 2.49 (2H, t, *J*=7.4 Hz, $-CH_2COO-$), 3.54-3.63 (8H, m, $-CH_2-N-CH_2-$), 3.82-3.90 (8H, br s, $-CH_2-O-CH_2-$), 4.74 (2H, s, $-OCH_2-CO-$), 7.57 (1H, dd, *J*=2.1 and 8.8 Hz, C7-H), 7.68 (1H, d, *J*=8.8 Hz, C8-H), 8.00 (1H, d, *J*= 2.1 Hz, C5-H), 8.06 (1H, br s, -NH-). *Anal.* Calcd for $C_{28}H_{41}N_5O_5$: C, 63.73; H, 7.83; N, 13.27. Found: C, 63.49; H, 7.90; N, 13.10.

6-(*O*-Dodecanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (5b)

mp 96-98 °C; yield 37 mg (98%); IR (KBr) 3304 (νN-H), 1748 (νC=O), 1673 (νC=O), and 1117 cm^{-1} (νC-O-C); ^1H NMR ($CDCl_3$, 270 MHz) δ=0.87 (3H, t, *J*=6.6 Hz, $-CH_3$), 1.22-1.42 (16H, m, $-(CH_2)_8-$), 1.65-1.77 (2H, m, $-CH_2CH_2COO-$), 2.49 (2H, t, *J*=7.6 Hz, $-CH_2COO-$), 3.53-3.63 (8H, m, $-CH_2-N-CH_2-$), 3.82-3.88 (8H, br s, $-CH_2-O-CH_2-$), 4.73 (2H, s, $-OCH_2-CO-$), 7.57 (1H, dd, *J*=2.2 and 8.8 Hz, C7-H), 7.68 (1H, d, *J*=8.8 Hz, C8-H), 8.00 (1H, d, *J*=2.2 Hz, C5-H), 8.04 (1H, br s, -NH-). *Anal.* Calcd for $C_{30}H_{45}N_5O_5 \cdot 0.2H_2O$: C, 64.42; H, 8.18; N, 12.52. Found: C, 63.30; H, 8.21; N, 12.43.

6-(*O*-Tetradecanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (5c)

mp: 99-100 °C; yield 33 mg (82%); IR (KBr) 3349 (νN-H), 1746 (νC=O), 1676 (νC=O), and 1112 cm^{-1} (νC-O-C); ^1H NMR ($CDCl_3$, 270 MHz) δ=0.85 (3H, t, *J*=6.5 Hz, $-CH_3$), 1.21-1.36 (20H, m, $-(CH_2)_{10}-$), 1.63-1.76 (2H, m, $-CH_2CH_2COO-$), 2.49 (2H, t, *J*=7.6 Hz, $-CH_2COO-$), 3.52-3.61 (8H, m, $-CH_2-N-CH_2-$), 3.80-3.87 (8H, br s, $-CH_2-O-CH_2-$), 4.72 (2H, s, $-OCH_2-CO-$), 7.57 (1H, dd, *J*=2.2 and 9 Hz, C7-H), 7.67 (1H, d, *J*=9 Hz, C8-H), 7.99 (1H, d, *J*=2.2 Hz, C5-H), 8.03 (1H, br s, -NH-). *Anal.* Calcd for $C_{32}H_{49}N_5O_5$: C, 65.84; H, 8.46; N, 12.0. Found: C, 65.7; H, 8.65; N, 11.84.

6-(*O*-Hexadecanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (5d)

mp 100-101 °C; yield 35 mg (82%); IR (KBr) 3303 (νN-H), 1741 (νC=O), 1676 (νC=O), and 1118 cm^{-1} (νC-O-C); ^1H NMR ($CDCl_3$, 270 MHz) δ=0.88 (3H, t, *J*=6.6 Hz, $-CH_3$), 1.22-1.38 (24H, m, $-(CH_2)_{12}-$), 1.66-1.78 (2H, m, $-CH_2CH_2COO-$), 2.49 (2H, t, *J*=7.5 Hz, $-CH_2COO-$), 3.56-3.60 (8H, m, $-CH_2-N-CH_2-$), 3.82-3.89 (8H, br s, $-CH_2-O-CH_2-$), 4.73 (2H, s, $-OCH_2-CO-$), 7.56 (1H, dd, *J*=2.3 and 8.8 Hz, C7-H), 7.68 (1H, d, *J*=8.8 Hz, C8-H), 7.99 (1H, d, *J*=2.3 Hz, C5-H), 8.03 (1H, br s, -NH-). *Anal.* Calcd for $C_{34}H_{53}N_5O_5 \cdot 0.1H_2O$: C, 66.55; H, 8.74; N, 11.41. Found: C, 66.38; H, 8.9; N, 11.27.

6-(*O*-Octadecanoylhydroxyacetyl)amino-2,3-dimorpholinoquinoxaline (5e)

mp 104-105 °C; yield 36 mg (81%); IR (KBr) 3303 (νN-H), 1742 (νC=O), 1676 (νC=O), and 1118 cm^{-1} (νC-O-C); ^1H NMR ($CDCl_3$, 270 MHz) δ=0.88 (3H, t, *J*=6.5 Hz, $-CH_3$), 1.21-1.39 (28H, m, $-(CH_2)_{14}-$), 1.66-1.78 (2H, m, $-CH_2CH_2COO-$), 2.49 (2H, t, *J*=7.6 Hz, $-CH_2COO-$), 3.56-3.60 (8H, m, $-CH_2-N-CH_2-$), 3.83-3.89 (8H, br s, $-CH_2-O-CH_2-$), 4.73 (2H, s, $-OCH_2-CO-$), 7.57 (1H, dd, *J*=2.5 and 8.8 Hz, C_7-H), 7.68 (1H, d, *J*=8.8 Hz, C_8-H), 8.00 (1H, d, *J*=2.5 Hz, C_5-H), 8.02 (1H, br s, -NH-). *Anal.* Calcd for $C_{36}H_{57}N_5O_5$: C, 67.57; H, 8.98; N, 10.94. Found: C, 67.36; H, 9.17; N, 10.88.

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