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SYNTHETIC HYDROGEN BONDING RECEPTORS AS MODELS OF TRANSACYLASE ENZYMES

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Abstract: A family of synthetic receptors has been prepared containing a barbiturate binding site and an appended thiol nucleophile. These are shown to cause large accelerations in the thiolysis reactions of barbiturate active ester derivatives. The size of the acceleration is shown to depend critically on the length and flexibility of the spacer that links the thiol to the receptor.

Introduction

The design of synthetic molecules that mimic key features of enzyme activity is an area of intense current interest. A simple approach involves the design of receptor molecules which contain a substrate binding site in close proximity to a reactive or catalytic group.[2] This strategy has been particularly effective in the development of transacylase mimics where a reactive nucleophile is linked to a complexation site in a position for nucleophilic attack on the carbonyl group of a non-covalently bound ester or amide substrate (Figure 1). Of the receptors containing this arrangement that have been reported, most have been based on either the solvophobic binding of aromatic esters into cyclodextrins[3], cyclophanes[4] and calixarenes[5] or the strongly

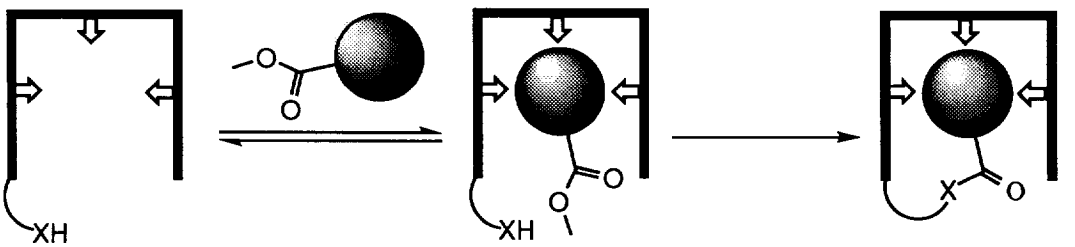


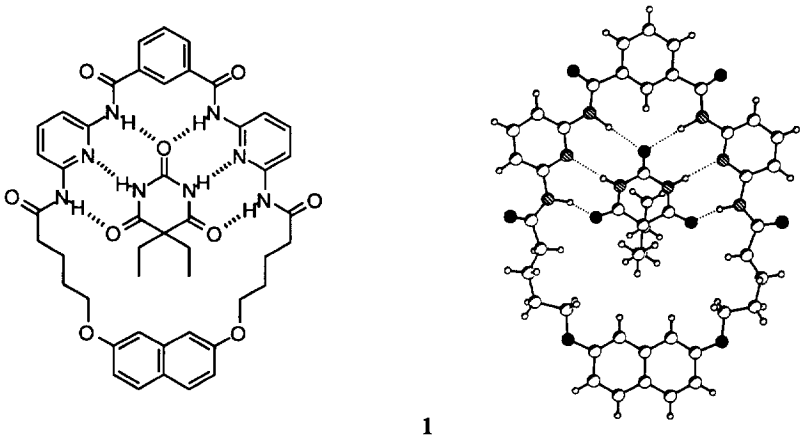
Figure 1. Schematic design of transacylase mimics.

electrostatic association of alkylammonium ions into functionalized crown ethers[6] or spherands.[7] In both classes the primary association involves non- or weakly directing forces leading to considerable uncertainty in the position of the substrate in the receptor. To overcome this, complex receptors or highly specialized substrates are required to achieve large rate accelerations. In the protease enzymes, however, multiple hydrogen bonds play a key role in the formation of the enzyme/substrate complex. These more directional interactions are

able not only to bind the substrate but also to orient it precisely with respect to catalytic groups in the active site. In this paper we report the design and synthesis of a family of synthetic receptors which achieve large accelerations in transacylase reactions by binding a peptide like substrate, solely via hydrogen bonds close to a linked nucleophilic group.[8] We also show that the size of the acceleration depends critically on the length and flexibility of the spacer that links the thiol to the barbiturate receptor site.

Receptor Design and Synthesis

We have previously shown that a molecule containing two 2,6-diaminopyridine units linked through an isophthalate spacer can form strong complexes with barbiturate substrates.[9] Extensive NMR and X-ray crystallographic studies have shown that six hydrogen bonds between the receptor and barbiturate are involved in forming a highly ordered complex, as in **1.** Our present goal was to exploit the strong binding in **1** to position an appropriate substrate within reaction distance of a functionalized receptor. Our plan was to incorporate nucleophiles onto the receptor in order to facilitate thiolysis of barbiturate-linked carboxylate ester substrates.



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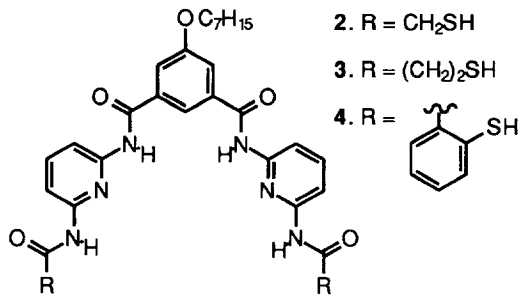
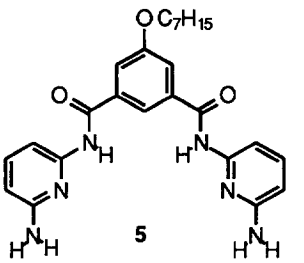
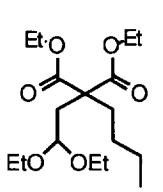
In modifying **1** we decided to simplify the synthesis by maintaining the two fold symmetry of the host. Thus, two identical nucleophiles were incorporated into the lower part of the host in anticipation that this would place them close to a bound barbiturate substrate containing an ester functionality in the 5-position. The thiol group was chosen as the attacking nucleophile since a range of derivatives is readily available synthetically. Also, thiols are good nucleophiles but poor hydrogen bond acceptors and should therefore not disrupt substrate binding.

A family of three receptors (**2**, **3**, and **4**) was synthesized with different lengths and flexibilities in the group linking the thiol to the receptor. All three were prepared from 1,3-bis[[(6-aminopyrid-2-yl)amino]carbonyl]-5-(heptyloxy)benzene **5** (itself formed by the reaction of 2,6-diaminopyridine with isophthaloyl dichloride[9]) by acylation with the benzoyl-protected mercaptoacid chloride derivative. Removal of the benzoyl group was achieved using aqueous NaOH. The substrate for these acyl transfer reactions was based on a derivative of 5,5-dialkyl barbituric acid which had been shown to bind strongly, as in **1**, to the bis-diaminopyridine receptors. It was anticipated that an ester at the end of one of the alkyl substituents would be held by the six hydrogen bonds in a position close to the receptor thiol group. A suitable acetate-substituted





barbiturate was prepared by alkylating diethyl butylmalonate with bromoacetaldehyde diethylacetal to form **6**. Reaction with urea and sodium ethoxide followed by gaseous HCl gave acetaldehyde-substituted barbiturate



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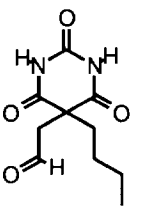

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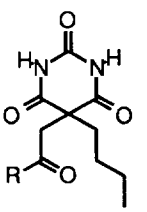
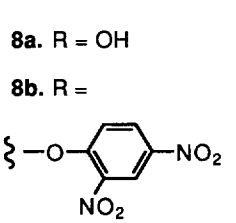
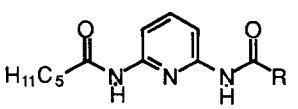
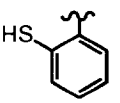


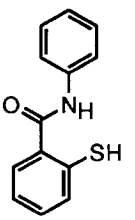


7 which was oxidized to the carboxylic acid **8a** using $KMnO_4$. The active ester **8b** was formed from a DCC coupling reaction of **8a** and 2,4-dinitrophenol. Reference 2,6-bis-(acylamino)pyridine derivatives **9** and **10**, corresponding to hosts with a thiol nucleophile but only three hydrogen bonding sites, were prepared by routes analogous to those for the larger systems.



9. R = CH_2SH **10**. R =





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Recognition-Induced Acceleration of Acyl Transfer Reactions

The simplified target reaction is outlined in Figure 2 and involves first binding of the barbiturate substrate followed by nucleophilic attack of the thiol on the activated ester. Breakdown of the tetrahedral intermediate will lead to the formation of the receptor-appended barbiturate derivative. In this present design no provision is made for the cleavage of the product thiol ester. Thus, a maximum of two turnovers is possible to form the bis-barbiturate derivative. The progress of the acyl transfer reactions was followed in CH_2Cl_2 by monitoring spectrophotometrically at 350 nm the release of 2,4-dinitrophenol. The thiol and lutidine, added as a

general base, were present in excess and the reactions were allowed to proceed through 5-6 half lives. Since the different thiols were unstable towards oxidation, especially under the mildly basic conditions of the reaction mixture, all kinetic studies were carried out in degassed solution. The rate plots were analyzed according to the first order expression, $(A_\infty-A_0)/(A_\infty-A_c)=e^{kt}$, using the Enzfitter program.[10]

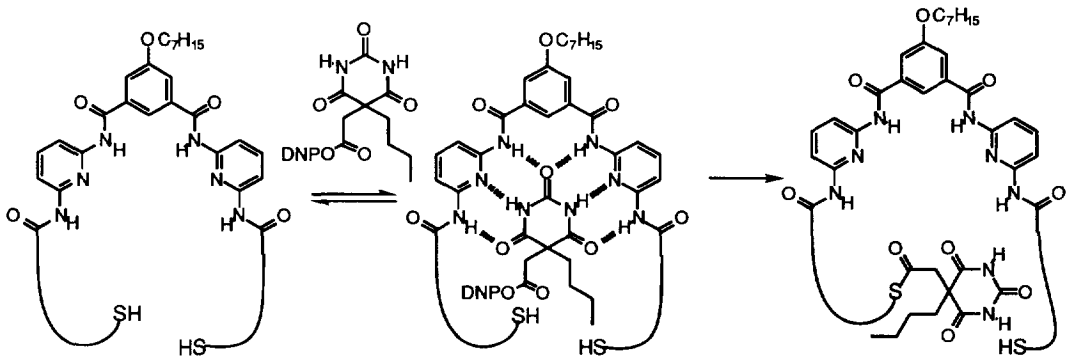

Figure 2. Receptor mediated thiolysis reaction.

Flexible Receptors 2 and 3.

Despite possessing a thiomethyl substituent on the lower acylaminopyridine, receptor **2** sites still binds strongly to barbiturate derivatives. Large downfield shifts of both pairs of amide protons were observed on titration of a $CDCl_3$ solution of **2** with substrate **8b** consistent with the formation of the six hydrogen bonded complex shown in Figure 2. Direct measurement of the K_a was complicated by the thiolysis reaction between **2** and **8b**. However, an estimate of $K_a \approx 2 \times 10^4\ M^{-1}$ in $CHCl_3$ can be made by comparison to the binding of diethyl barbiturate to an acyclic analog of **1**.[9] Pseudo-first order rate constants for the thiolysis of **8b** by different concentrations of **2** are collected in Table 1. These show that at 0.24 mM **2** causes a 220 fold acceleration compared with ethyl mercaptoacetate (at 0.46 mM), chosen as a control that contains a thiol of

Table 1. Pseudo-first order rate constants for reaction of **8b** with thiomethyl derivatives.

Catalyst	[Cat] x 10^4 (M)	[Cat]/[8b]	k_{obs} (sec^{-1})	k_{obs}/k_o
$HSCH_2CO_2Et$	4.64	21	6.40×10^{-6}	1
9	4.64	21	3.91×10^{-5}	61
2	2.42	11	1.41×10^{-3}	220
2	2.90	13	1.67×10^{-3}	260
2	3.39	15	1.83×10^{-3}	285
2	3.88	17.4	1.94×10^{-3}	300
2	4.36	19.5	2.15×10^{-3}	335

similar pK_a but without a binding site.[11] An Eadie-Hofstee plot gave a K_m of $1.4 \times 10^3\ M^{-1}$ and a k_{cat} of $5.7 \times 10^{-3}\ sec^{-1}$ for the reaction between **8b** and **2**. The reduced binding affinity, compared to that estimated above,

reflects the presence of 0.043 M lutidine in the reaction mixture. Under comparable conditions bis-(acylamino)pyridine **9** (at 0.46 mM) accelerated the reaction by 61-fold.

This relatively small increase in efficiency of **2** compared to **9**, despite a much larger K_a for binding barbiturate derivatives (≈2 x 10^4 vs. 1 x 10^2 M^{-1} in $CDCl_3$),[9] suggested an imperfect positioning of the thiol group relative to the substrate binding site. Indeed, molecular modeling on the complex between **2**:**8b** indicated that the methylene spacer in **2** was too short to optimally position the thiol for reaction with the active ester. A better fit is provided by a two carbon spacer between the acylaminopyridine and thiol and consequently thiopropionate **3** was tested in the thiolysis reaction. Surprisingly, the thiolysis reaction of **3** was very slow compared to **2**, requiring the use of the initial rates method. An Eadie-Hofstee plot gave a K_m = 1.2 x 10^3 M^{-1} and k_{cat} = 2.2 x 10^{-4} sec^{-1}. The binding constant for **3**:**8b** is very similar to that measured for **2**:**8b**, however the k_{cat} value is much slower (2.2 x 10^{-4} sec^{-1} vs. 5.7 x 10^{-3} sec^{-1}). Furthermore, at 0.4 mM **3** causes a marginal 1.3 fold acceleration compared with ethyl mercaptopropionate (at 0.84 mM)[11] (Table 2).

Table 2. Pseudo-first order rate constants for reaction of **8b** with thioethyl derivatives.

Catalyst	[Cat] x 10^4 (M)	[Cat]/[8b]	k_{obs} (sec^{-1})	k_{obs}/k_o
$HSCH_2CH_2CO_2Et$	8.4	38	5.50 x10^{-5}	1
3	3.5	16	6.74 x 10^{-5}	1.2
3	4.0	18	7.20 x 10^{-5}	1.3
3	4.5	20.5	7.69 x 10^{-5}	1.4
3	5.5	25	8.91 x 10^{-5}	1.6
3	6.5	29.5	9.73 x 10^{-5}	1.8
3	7.5	34	1.06 x 10^{-4}	1.9

Because of the differences in the acidity of their thiol groups, direct comparison of the efficiency of **2** and **3** in accelerating the thiolysis reaction is difficult. However, the relative rate acceleration measured against the control thiols[11] (220-fold for **2** and 1.3-fold for **3**) points to a flawed design in **3**. The results suggest that any advantage of proximity is lost by increasing the conformational freedom of the nucleophile in **3**. This is consistent with Breslow's observation[12] of a 9-fold increase in rate of a related acyl transfer reaction when the rotation around one bond in the substrate is frozen.

Rigid Receptor 4

The 2-thiobenzoyl receptor **4** was designed to maintain the approximate nucleophile/electrophile distance of the propionate host **3** but with increased rigidity of the side arm. Figure 3 shows a calculated structure[13] for the complex between bis-(2-thiobenzoyl) receptor **4** and the phenyl ester of **8a**. The six hydrogen bonded complex positions the thiol group close (3.33Å) to the carbonyl carbon of the substrate ester. Furthermore, the S-C-O angle is close to the optimum trajectory for acyl transfer to the thiol group. Receptor **4** was so effective in the thiolysis of **8b** that the rate was too fast to measure by simple methods. It was necessary to lower the concentration of 2,6-lutidine from 0.043 M to 0.0043 M. This makes any comparison with the previous systems difficult and for this reason **4** is treated separately in the present section. Table 3 contains the

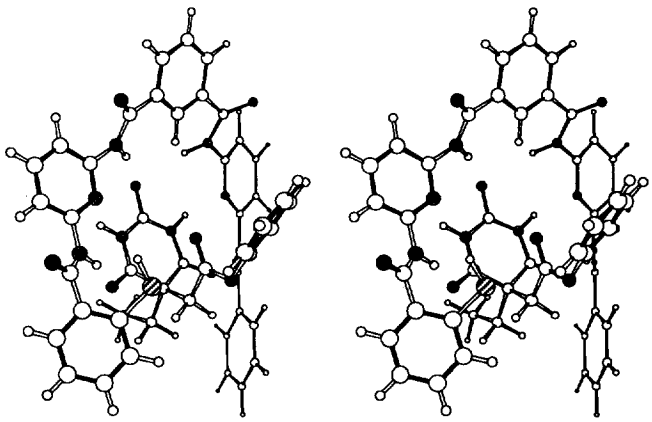

Figure 3. Stereoview of the calculated[13] structure for the complex between **4** and phenyl ester of **8a**.

measured k_{obs} values for different concentrations of **4** and shows that at 0.40 mM this more rigid host causes an 11,700-fold increase in the rate of the transacylation reaction when compared to 2-mercaptobenzanilide **11** at 0.88 mM.[14] Figure 4 shows the dependence of the rate constant for the thiolysis of **8b** on the concentration of receptor **4**.

Table 3. Pseudo-first order rate constants for reaction of **8b** with 2-thiobenzoyl derivatives.

Catalyst	[Cat] x 10^4 (M)	[Cat]/[8b]	k_{obs} (sec^{-1})	k_{obs}/k_o
11	8.84	38	6.50×10^{-6}	1
10	4.40	20	5.78×10^{-5}	9
4	0.57	2.5	3.95×10^{-2}	6,100
4	1.13	5	5.46×10^{-2}	8,400
4	2.27	10	6.82×10^{-2}	10,500
4	4.54	20	7.58×10^{-2}	11,700
4	5.68	25.5	7.88×10^{-2}	12,100

The reaction follows Michaelis-Menten kinetics and from an Eadie-Hofstee analysis (Figure 5) a K_m of 1.4×10^4 M^{-1} can be calculated. This value is in agreement with the binding constant estimated for **4** and diethyl barbiturate in $CDCl_3$ (2.0×10^4 M^{-1}), showing that at a concentration of 4.3 mM the lutidine does not inhibit substrate binding. This analysis also gives a k_{cat} value of 8.79×10^{-2} sec^{-1}, corresponding to the maximum value of the pseudo-first order rate constant attainable under the experimental conditions when all the substrate is bound to the receptor. That strong binding is a requirement for this effect is confirmed by the small acceleration (9 fold) seen with the simple bis-(acylamino)pyridine **10** which contains only three hydrogen bonding sites.

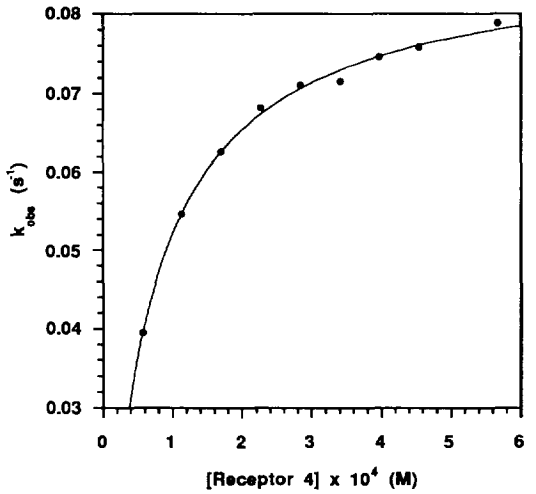

Figure 4. Dependence of k_{obs} for the thiolysis of **8b** on the concentration of **4**.

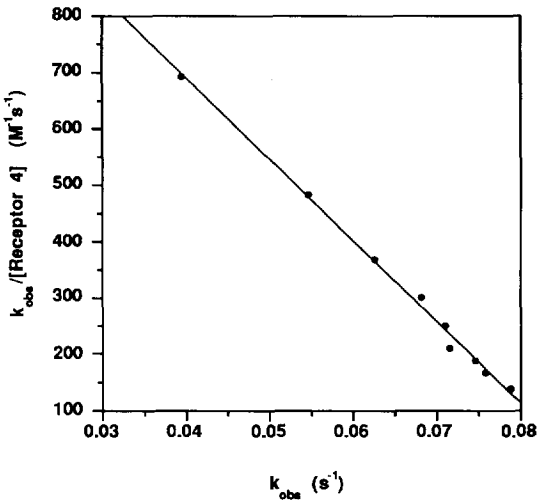

Figure 5. Eadie-Hofstee plot for the reaction between **4** and **8b**

The previous reactions have all been carried out in the presence of a large excess of 2,6-lutidine. In order to assess the role of the base in the receptor-mediated reaction, a series of experiments in which the lutidine concentration was varied from 4.3-430 mM was investigated. A plot of k_{obs} versus lutidine concentration (Figure 6a) shows saturation behavior with an essentially linear relationship at low lutidine concentrations. A plot of log k_{obs} versus log[lutidine] gave a straight line with a slope of 0.96 which indicates that the thiolysis reaction is first order in lutidine, involving one molecule of the base interacting with the thiol nucleophile. Similar behavior was seen in the control reaction between 2-mercaptobenzanilide **11** and **8b**

(Figure 6b). At concentrations of lutidine >0.1 M the rate increase levels off in both reactions, consistent with the formation of a discrete complex between the receptor and lutidine. In the case of the receptor-mediated reaction (Figure 6a) there is a downward curvature in the plot at lutidine concentrations >0.25 M. This decrease in reaction rate with increasing concentrations of base presumably reflects the disruption of hydrogen bonding between the barbiturate and diamiopyridine units that occurs in more polar solvents.

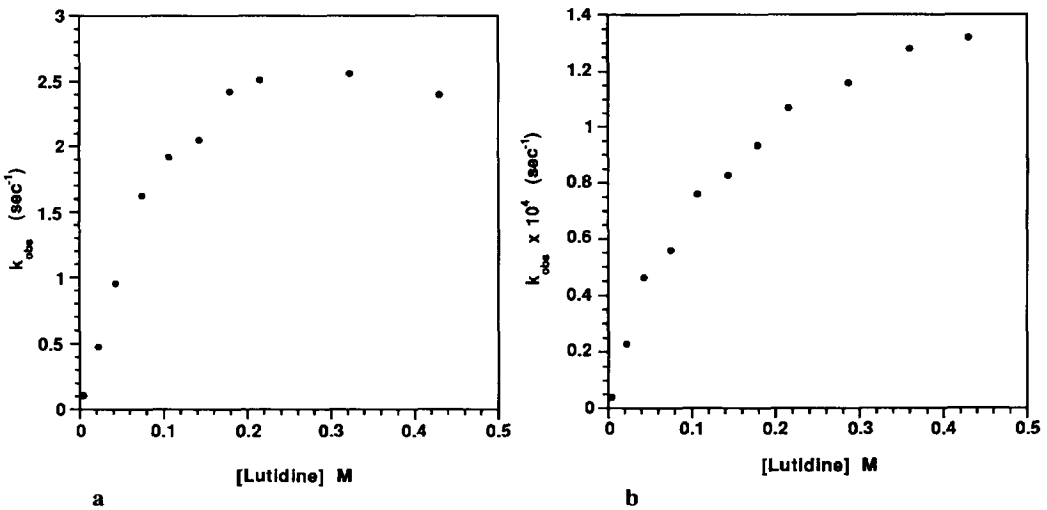

Figure 6. Effect of varying lutidine concentrations in CH_2Cl_2 at 25 °C on the rate of thiolysis of **a) 8b** (5.5 x 10^{-5} M) and **4** (4.4 x 10^{-4} M) and **b) 8b** (5.5 x 10^{-5} M) and **11** (8.8 x 10^{-4} M).

In summary, we have shown that a synthetic receptor that binds strongly to barbiturates via multiple hydrogen bonds can be prepared containing appended thiol nucleophiles. This design does not cause a disruption in substrate binding but instead leads to large rate accelerations in the thiolysis of complementary barbiturate ester derivatives. The size of the acceleration is dependent on the length and flexibility of the spacer that links the thiol to the receptor.

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Experimental Section.

General Methods and Materials. Melting points are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AF300 spectrometer operating at 300.13 MHz, and chemical shifts are reported relative to internal Me_4Si. 2,6-Lutidine, ethyl 2-mercaptoacetate, ethyl 3-mercaptopropionate were Aldrich products and were distilled before use. 2-Mercapto-N-phenylbenzamide was prepared and purified by literature methods.[15]

2-(Benzoylmercapto)ethanoic acid. To a solution containing $NaHCO_3$ (2.6g, 0.031 mol) in water (25 mL) at

0 °C was added dropwise mercaptoacetic acid (2.0 g, 0.022 mol) followed by benzoyl chloride (3.05 g, 0.022 mol). After the additions were complete, further $NaHCO_3$ (2.5 g, 0.03) was added. The mixture was stirred for 15 minutes at 0 °C and for an additional 15 minutes at room temperature. The mixture was acidified with concentrated HCl and the white precipitate was collected, washed with cold water, and dried in a vacuum desiccator. Recrystallization from CH_2Cl_2/hexanes afforded 3.1 g (72 %) of 2-(benzoylmercapto)ethanoic acid: Mp: 103-104 °C. (lit: 105.5-106[16]); ^{1}H NMR ($CDCl_3$): 3.90 (s, 2 H, CH_2COOH), 7.47 (t, J = 7.7 Hz, 2 H, $H_{3,5}$Ph), 7.60 (t, J = 7.7 Hz, 1 H, H_4Ph), 7.96 (d, J = 7.7 Hz, 2 H, $H_{2,6}$Ph), 8.00 (bs, 1 H, COOH).

1,3-Bis[[[6-(2-(benzoylmercapto)acetylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene. To a solution of 2-(benzoylmercapto)ethanoic acid (0.186 g, 0.95 mmol) in dry CH_2Cl_2 (10 mL) was added oxalyl chloride (0.5 mL, 5.73 mmol) and a drop of DMF. The slurry was stirred at room temperature until a clear solution was obtained. The solvent was evaporated under reduced pressure and the residue was dissolved in dry THF (10 mL). To this solution were added 1,3-bis[[(6-aminopyrid-2-yl)amino]carbonyl]-5-(heptyloxy)benzene **5**[17] (0.200 g, 0.43 mmol) and triethylamine (0.094g, 0.83 mmol). The solution was stirred overnight at room temperature under an inert atmosphere. The solvent was evaporated and the residue was dissolved in CH_2Cl_2. The organic phase was washed several times with a saturated solution of $NaHCO_3$, dried over Na_2SO_4 and evaporated to afforded the crude product which was purified by flash column chromatography (silica gel, 1% CH_3OH/CH_2Cl_2) and crystallized (CH_2Cl_2/hexanes) to give 0.200 g (56%) of 1,3-Bis[[[6-(2-(benzoylmercapto)acetylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene. Mp: 110-120 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 6.6 Hz, 3 H, $(CH_2)_6CH_3$), 1.32 (m, 6 H, $(CH_2)_3(CH_2)_3CH_3$), 1.48 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$) 1.82 (m, 2 H, $CH_2CH_2(CH_2)_3CH_3$), 4.02 (s, 4 H, CH_2S) 4.08 (b, J = 6.4 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 7.37 (t, J = 7.9 Hz, 4 H, $H_{3,5}$PhCO), 7.55 (t, J = 7.9 Hz, 2 H, H_4PhCO), 7.69 (s, 2 H, $H_{2,6}$isophth), 7.72 (d, J = 8.0 Hz, 2 H, H_4Py); 7.90 (d, J = 8.0 Hz, 2 H, H_3 Py), 7.92 (d, J = 7.90 Hz, 4 H, $H_{2,6}$Ph), 8.04 (d, J = 8.0 Hz, 2 H, H_5 Py), 8.16 (s, 1 H, H_4isophth), 8.77 (bs, 2 H, NH), 8.82 (bs, 2 H, NH); MS (FAB) *m/e* calcd. for $C_{43}H_{42}N_6O_7S_2$: 818.76, found 819.

1,3-Bis[[[6-(2-mercaptoacetylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene (2). The foregoing benzoyl protected receptor (0.10 g, 0.12 mmol) was dissolved in THF (5 mL) and MeOH (10 mL). A solution of 0.2 M NaOH (2.5 mL) was added and the mixture was stirred for 1 h at room temperature, under an inert atmosphere. A saturated solution of $NaHCO_3$ (20 mL) was then added and the aqueous layer was extracted with CH_2Cl_2 (3 x 25 mL). The organic phase was dried over Na_2SO_4 and evaporated to give a solid residue which was recrystallized from CH_2Cl_2/hexanes to afford 0.067g (90%) of **2**. Mp: 166-167 °C. ^{1}H NMR ($CDCl_3$) δ 0.91 (t, J = 6.7 Hz, 3 H, $(CH_2)_6CH_3$), 1.34 (m, 6 H, $(CH_2)_3(CH_2)_3CH_3$), 1.50 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$), 1.84 (m, 2H, $CH_2CH_2(CH_2)_4CH_3$), 3.44 (s, 4H, CH_2S), 4.105 (t, J = 6.5 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 7.65 (s, 2 H, $H_{4,6}$Ph), 7.81 (t, J = 8.05 Hz, 2 H, H_4Py), 7.96 (d, J = 8.1 Hz, 2 H, H_3Py), 7.80 (s, 1 H, H_2Ph), 8.11 (d, J = 8.1 Hz, 2 H, H_5Py), 8.49 (bs, 2 H, NHCO), 8.89 (bs, 2 H, NHCO); MS (FAB) *m/e* calcd. for $C_{29}H_{34}N_6O_5S_2$: 610.56, found 611.

3-(Benzoylmercapto)propionic acid. To a solution of containing Na_2CO_3 (2.92 g, 0.027 mol) in water (40 mL) at 0 °C was added mercaptopropionic acid (2.43 g, 0.023 mol) and benzoyl chloride (3.23 g, 0.023 mmol). The reaction mixture was stirred for 15 minutes at 0 °C and for an additional 1 h at room temperature. The mixture was acidified with concentrated HCl and extracted with Et_2O. The organic phase was dried over Na_2SO_4 and evaporated to afford a clear oil that crystallized on standing. Recrystallization from

CH_2Cl_2/hexanes gave 2.83 g (59%) of 3-(benzoylmercapto)propionic acid: Mp: 49-50 °C (lit[18]: 48-50). ^1H NMR ($CDCl_3$) δ 2.81 (t, J = 7.1 Hz, 2 H, CH_2CH_2COOH), 3.31 (t, J = 7. Hz, 2 H, CH_2CH_2COOH), 7.45 (t, J = 7.1 Hz, 2 H, $H_{3,5}$Ph), 7.58 (tt, J = 7.1 and 1.6 Hz, 1 H, H_4Ph), 7.96 (dd, J = 7.1 and 1.6 Hz, 2 H, $H_{2,6}$Ph).

1,3-Bis[[[6-(3-(benzoylmercapto)propionylamino)pyrid-2-yl]amino] carbonyl]-5-(heptyloxy)benzene. This was prepared in a similar way to 1,3-bis[[[6-((2-benzoylmercapto)acetylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene as described above using 3-(benzoylmercapto)propionic acid (0.25 g, 1.2 mmol) in dry CH_2Cl_2 (25 ml) and oxalyl chloride (0.5 mL, 5.73 mmol) and a drop of DMF. Reaction of the acid chloride in dry THF (20 mL) with 1,3-bis[[(6-aminopyrid-2-yl)amino]carbonyl]-5-(heptyloxy)benzene[9] (0.25 g, 0.54 mmol) and triethylamine (0.170 mL) gave after flash column chromatography (silica gel, 5% MeOH/CH_2Cl_2) and crystallization from CH_2Cl_2/hexanes 0.23 g (50%) of 1,3-Bis[[[6-(3-benzoylmercapto)propionylamino)-pyrid-2-yl]amino] carbonyl]-5-(heptyloxy)benzene. Mp: 88-89 °C. ^1H NMR ($CDCl_3$) δ 0.89 (t, J = 6.7 Hz, 3 H, $(CH_2)_6CH_3$), 1.30 (m, 6 H, $(CH_2)_2(CH_2)_3CH_3$), 1.45 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$), 1.79 (m, 2 H, $CH_2CH_2(CH_2)_4CH_3$), 2.82 (bt, J = 6.7 Hz, 4 H, CH_2CONH), 3.40 (t, J = 6.7 Hz, 4 H, PhC(O)SCH_2CH_2), 4.03 (t, J = 6.5 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 7.40 (t, J = 7.8 Hz, 4 H, $H_{3,5}$PhCO), 7.54 (t, J = 7.3 Hz, 2 H, H_4PhCO), 7.63 (s, 2 H, $H_{4,6}$isophth), 7.73 (t, J = 8.0 Hz, 2 H, H_4Py), 7.92 (m, 6 H, H_3Py and $H_{2,6}$PhCO), 8.02 (m, 5 H, H_3Py, H_2isophth and NHC(O)), 8.56 (bs, 2 H, NHC(O)); MS (FAB) *m/e* calcd. for $C_{45}H_{46}N_6O_7S_2$: 846.82, found 847.

1,3-Bis[[[6-(3-mercaptopropionylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene (3). This was prepared using the same method as **2**. The foregoing benzoyl derivative (0.10 g, 0.12 mmol) was reacted in THF (10 mL) and MeOH (10 mL) with a solution of 0.2 M NaOH (5 mL). Work up and recrystallization from CH_2Cl_2/hexanes afforded 0.50 g (66%) of **3**. Mp: 114-115 °C. ^1H NMR ($CDCl_3$) δ 0.90 (m, 3 H, $(CH_2)_6CH_3$), 1.32 (m, 6 H, $(CH_2)_3(CH_2)_3CH_3$), 1.49 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$), 1.76 (t, J = 7.2 Hz, 2 H, SH), 1.84 (m, 2 H, $CH_2CH_2(CH_2)_4CH_3$), 2.78 (t, J = 7.2 Hz, 4 H, $HSCH_2CH_2C(O)NH$), 2.94 (m, 4 H, $HSCH_2CH_2C(O)NH$), 4.10 (t, J = 6.5 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 7.65 (s, 2 H, $H_{4,6}$Ph), 7.84 (t, J = 8.0 Hz, 2 H, H_4Py), 8.04 (m, 5 H, H_2Ph, H_5Py , and NHC(O)), 8.12 (d, J = 8.0 Hz, 2 H, H_3Py), 8.56 (bs, 2H, NHC(O)); MS (FAB) *m/e* calcd. for $C_{31}H_{38}N_6O_5S_2$: 638.61, found 639.

1,3-Bis[[[6-(2-(benzoylmercapto)benzoylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene. This was prepared as for the aliphatic derivatives above from 2-mercaptobenzoic acid (3.32 g, 0.021 mmol), Na_2CO_3 (3.62 g, 0.034 mol) in water (100 mL) and benzoyl chloride (2.5 mL, 21 mmol) to form, after recrystallization from EtOH, 4.1 g (74%) of 2-(benzoylmercapto)benzoic acid. Mp: 162-164 °C (lit [19]:161-163 °C). ^1H NMR ($CDCl_3$) δ 7.42-7.73 (m, 6 H, $H_{3,4,5}$PhS and $H_{3,4,5}$PhCO), 8.03 (dt, J = 7.0 and 1.5 Hz, 2 H, $H_{2,6}$PhCO), 8.12 (dt, J = 7.0 and 1.9 Hz, 1 H, H_6PhS).

The 2-(benzoylmercapto)benzoic acid (0.50 g, 1.9 mmol) in dry CH_2Cl_2 (20 mL) was converted with oxalyl chloride (1 mL, 11.5 mmol) into the acid chloride. This was further treated in dry THF (20 mL) with 1,3-bis[[(6-aminopyrid-2-yl)amino]carbonyl]-5-(heptyloxy)benzene **5**[9] (0.30 g, 0.65 mmol) and triethylamine (0.145 g, 1.27 mmol) to give after workup and recrystallization from CH_2Cl_2/hexanes 0.45 g (73%) of 1,3-bis[[[6-(2-(benzoylmercapto)benzoylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene. Mp: 128-130 °C. ^1H NMR ($CDCl_3$) δ 0.91 (t, J = 7.0 Hz, 3 H $(CH_2)_2CH_3$), 1.33 (m, 6 H, $(CH_2)_3(CH_2)_3CH_3$), 1.49 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$), 1.84 (m, 2 H, $CH_2CH_2(CH_2)_4CH_3$), 4.07 (t, J = 6.5 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 7.31 (m, 12 H, $H_{3,4,5}$PhS and $H_{3,4,5}$PhCO), 7.57 (m, 6 H, H_4Py, $H_{4,6}$isophth, and H_6PhS), 7.87 (m, 9 H, $H_{3,5}$Py,

$H_{2,6}$PhCO, and H_2isophth), 8.54 (bs, 2 H, NH), 8.70 (bs, 2 H, NH); MS (FAB) *m/e* calcd. for $C_{53}H_{46}O_7N_6S_2$: 942.28, found 943. Anal Calcd. for $C_{53}H_{46}O_7N_6S_2$: C, 67.50; H, 4.92; N, 8.92. Found: C, 66.50; H, 4.91; N, 8.96.

1,3-Bis[[[6-(2-mercaptobenzoylamino)pyrid-2-yl]amino]carbonyl]-5-(heptyloxy)benzene (4). This was prepared by a method similar to that for **2**. The foregoing benzoyl derivative (0.05 g, 0.07 mmol) in THF (5 ml) and MeOH (10 mL) was treated with 0.2 M NaOH (2 mL). Workup and recrystallization from CH_2Cl_2/hexanes gave 0.035 g (90 %) of **4**. Mp: 113-115 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 6.7 Hz, 3 H, $(CH_2)_6CH_3$), 1.32 (m, 6 H, $(CH_2)_3(CH_2)_3CH_3$), 1.46 (m, 2 H, $(CH_2)_2CH_2(CH_2)_3CH_3$), 1.82 (m, 2 H, $CH_2CH_2(CH_2)_4CH_3$), 4.07 (t, J = 6.4 Hz, 2 H, $CH_2(CH_2)_5CH_3$), 4.46 (s, 2 H, SH), 7.2 (t, J = 7.8 Hz, 2 H, H_5PhS), 7.33 (7, J = 7.8 Hz, 2 H, H_4PhS), 7.37 (t, J = 7.8 Hz, 2 H, H_3PhS) 7.61 (m, 4 H, $H_{4,6}$isophth and H_6PhS), 7.81 (t, J =8.04 Hz, 2 H, H_4Py), 7.98 (s, 1 H, H_2isophth), 8.09 (m, 4 H, $H_{3,5}$Py), 8.30 (bs, 2 H, NH), 8.54 (bs, 2 H, NH); MS (FAB) *m/e* calcd. for $C_{39}H_{38}N_6O_5S_2$: 734.89, found 735.

2-Amino-6-hexanoylaminopyridine. To a solution of 2,6-diaminopyridine (2.5 g, 0.023 mol) in dry THF (250 ml) was added dropwise over a period of 2 h a solution containing hexanoyl chloride (6.6 g, 0.046 mol) in dry THF (250 ml). After the addition was completed the reaction mixture was stirred for 18 h at room temperature under argon The solvent was evaporated and the residue was taken up with CH_2Cl_2 (250 ml) and washed with a saturated solution of $NaHCO_3$(10 x 100 ml). The organic layer was dried (Na_2SO_4) and evaporated to afford 9.0 g (94 %) of 2-amino-6-hexanoylaminopyridine. Mp: 81-82 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 6.6 Hz, 3 H, $(CH_2)_4CH_3$), 1.34 (m, 4 H, $(CH_2)_2(CH_2)_2CH_3$), 1.703 (m, 2 H, $CH_2CH_2(CH_2)_2CH_3$), 2.33 (t, J = 7.5 Hz, 2 H, $CH_2(CH_2)_3CH_3$), 4.21 (bs, 2 H, NH_2), 6.24 (d, J = 7.7 Hz, 1 H, H_3Py), 7.44 (t, J = 7.7 Hz, 1 H, H_4Py), 7.54 (d, J = 7.7 Hz, 1 H, H_5Py), 7.59 (bs, 1 H, CONH); MS calcd. for $C_{11}H_{17}N_3O$: 207.1372, found 207.1372. *m/e* 207 (M^+), 109 (100%), 93 (10%), 82 (15 %). Anal. Calcd. for $C_{11}H_{17}N_3O$: C, 63.74; H, 8.27; N, 20.21. Found: C, 63.90; H, 8.49; N, 20.34.

2-(2-(Benzoylmercapto)acetylamino)-6-hexanoylaminopyridine. To a solution of 2-amino-6-hexanoylaminopyridine (0.190 g, 0.092 mmol) in dry THF (10 mL) was added 2-(benzoylmercapto)ethanoyl chloride (0.235 g, 1.02 mmol) and dry triethylamine (0.109 g, 0.954 mmol). The reaction was stirred for 18 h at room temperature under argon. The solvent was then evaporated and the residue dissolved in CH_2Cl_2. The organic solution was washed with a saturated solution of $NaHCO_3$, dried (Na_2SO_4) and evaporated to give the crude product which was purified by flash column chromatography (silica gel, 2.5% MeOH/CH_2Cl_2) and recrystallized from CH_2Cl_2/hexanes to afford 0.290 g (82 %) of the pale yellow 2-(2-(Benzoylmercapto)-acetylamino)-6-hexanoylaminopyridine. Mp: 121-122 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J =7.1 Hz, 3 H, $(CH_2)_4CH_3$), 1.35 (m, 4 H, $CH_2(CH_2)_2CH_3$), 1.74 (m, 2 H, $CH_2CH_2(CH_2)_2CH_3$), 2.36= 7.1 Hz, 2 H, $CH_2(CH_2)_4CH_3$), 3.88 (s, 2H, CH_2S),7.53 (t, J = 8.2, 2 H, $H_{3,5}$PhCO), 7.62 (bs, 1 H, $NHC(O)C_5H_{11}$), 7.67 (t, J = 8.2, 1 H, H_4PhCO), 7.69 (t, J = 7.7 Hz,1 H, H_4Py), 7.85 and 7.92 (2d, J = 7.7 Hz, 2 H, $H_{3,5}$Py), 8.02 (d, J =8.25, 2 H, $H_{2,6}$PhCO), 8.54 (bs, 1H, $NHC(O)CH_2S$); MS M^+ calcd. for $C_{20}H_{23}N_3O_3S$: 385.1460, found 385.1460, *m/e* 385 (30%, M^+), 280 (100%, (M - PhCO)$^+$), 182 (30%), 150 (10%), 136 (15 %), 105 (70%, $PhCO^+$), 77 (20%).

2-(Mercaptoacetylamino)-6-hexanoylaminopyridine (9). The foregoing benzoyl derivative (0.050 g, 0.13 mmol) in dioxane (5 mL) was deprotected as above using 0.2 M NaOH (1.5 ml). Workup and recrystallization from CH_2Cl_2/hexanes gave 0.030 g of pale **9**. Mp: 104-105 °C. ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 3 H, $(CH_2)_4CH_3$),

1.36 (m, 4 H, $(CH_2)_2(CH_2)_2CH_3$), 1.73 (m, 2 H, $CH_2CH_2(CH_2)_2CH_3$), 2.02 (t, J = 9.3 Hz, 1 H, SH), 2.38 (t, J = 7.7 Hz, 2 H, $CH_2(CH_2)_3CH_3$), 3.47 (d, J = 9.3, 2 H, CH_2SH), 7.58 (bs, 1 H, $NHC(O)(CH_2)_5CH_3$), 7.72 (t, J = 8.2 Hz, 1 H, H_4 Py), 7.88 and 7.96 (2d, J = 8.2 Hz, 2 H, $H_{3,5}$Py), 8.75 (bs, 1 H, $NHC(O)CH_2SH$); MS M^+ calcd. $C_{13}H_{19}N_3O_2S$: 281.1198, found 281.1198, *m/e* 281 (50%, M^+), 234 (100%), 208 (50%), 183 (20%), 136 (70%), 109 (30%).

2-(2-(Benzoylmercapto)benzoylamino)-6-hexanoylaminopyridine. This was prepared as above from 2-(benzoylmercapto)benzoic acid (0.75 g, 2.90 mmol) in dry CH_2Cl_2 (50 mL) and oxalyl chloride (2mL) and 1 drop of DMF. The resuting acid chloride was reacted in dry THF (50 mL) with 2-amino-6-hexanoylaminopyridine (0.5 g, 2.40 mmol) and triethylamine (0.5 ml). Workup and crystallization from THF/hexanes gave 0.4 g (37%) of 2-(2-(benzoylmercapto)benzoylamino)-6-hexanoylaminopyridine. Mp: 57-58 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 7.0 Hz, 3 H, $(CH_2)_4CH_3$), 1.31 (m, 4 H, $(CH_2)_2(CH_2)_2CH_3$), 1.67 (q, J = 7.0 Hz, 2 H, $CH_2CH_2(CH_2)_2CH_3$), 2.28 (t, J = 7.0 Hz, 2 H, $CH_2(CH_2)_3CH_3$), 7.40-7.76 (m, 9 H, $H_{3,4,5}$PhCO $H_{3,4,5}$PhS, $H_{3,4}$Py, and NH), 7.96 (m, 4H , $H_{2,6}$ PhCO H_2PhS, and H_5Py), 8.48 (bs, 1 H, NH); MS M^+ calcd. for $C_{25}H_{25}N_3O_3S$: 447.1617, found 447.1617, *m/e* 447 (10%, M^+), 342 (15%), 311 (25%), 268 (10%), 255 (10%), 213 (40%) 185 (30%), 105 (100%), 77 (50%). Anal. Calcd. for $C_{25}H_{25}N_3O_3S$: C, 67.09; H, 5.63; N, 9.39. Found: C, 66.71; H, 5.64; N, 9.27.

2-(2-Mercaptobenzoylamino)-6-hexanoylaminopyridine (10). This was prepared as above from the foregoing benzoyl derivative (0.40g, 0.90 mmol) in THF (5 mL) and CH_3OH (15 mL) with 0.1 M NaOH (2.5 mL). Workup and crystallization from THF/hexanes gave 0.28 g (91%) of **10**. Mp 43-45 °C. ^{1}H-NMR ($CDCl_3$) δ 0.90 (t, J = 6.9 Hz, 3 H, $(CH_2)_4CH_3$), 1.37 (m, 4 H, $(CH_2)_2(CH_2)_2CH_3$), 1.74 (m, 2 H, $CH_2CH_2(CH_2)_2CH_3$), 2.38 (t, J = 7.5 Hz, $CH_2(CH_2)_3CH_3$), 4.50 (s, 1 H, SH), 7.25 (dd, J = 9.2 and 1.3 Hz, 1 H, H_3PhS), 7.38 (m, 2 H, $H_{4,5}$PhS), 7.56 (bs, 1H, NH), 7.61 (dd, J = 7.7 and 1.32 Hz, 1 H, H_6PhS), 7.77 (t, J = 8.1 Hz, 1 H, H_4Py), 7.98 (d, J = 8.0 Hz, 1 H, H_5Py), 8.03 (d, J = 8.0 Hz, 1 H, H_3Py), 8.14 (bs, 1 H, NH). Anal. Calcd. for $C_{18}H_{21}N_3O_2S$: C, 62.96; H, 6.16; N, 12.23. Found: C, 62.85; H, 6.18; N, 12.15.

Ethyl 2-ethoxycarbonyl-2-(2,2-diethoxyethyl)hexanoate (6). To a suspension of potassium tert-butoxide (3.0 g, 0.027 mol) in 30 ml of dry toluene (30 mL) was added ethyl butylmalonate.[20] The slurry was stirred at room temperature until all the potassium tert-butoxide had gone into solution (approx. 10 min). To this solution, bromoacetaldehyde diethylacetal (6.3 g, 0.032 mol) was added and the mixture was heated at reflux overnight. After cooling, 5 % aqueous HCl was added and the aqueous layer was extracted with CH_2Cl_2 (3 x 50 ml). The organic extracts were combined, dried (Na_2SO_4) and evaporated to give a yellow liquid which was purified by fractional distillation at reduced pressure to afford 4.2 g (47 %) of colorless **6**. Bp: 115-120 °C (0.5 mm/Hg). ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 7.3 Hz, 3 H, $(CH_2)_3CH_3$), 1.17 (t, J = 7.3 Hz, 6 H, $CH(OCH_2CH_3)_2$), 1.23 (t, J = 7.1 Hz, 6 H, $C(O)OCH_2CH_3$), 1.28 (m, 4 H, $CH_2(CH_2)_2CH_3$), 1.92 (m, 2 H, $CH_2(CH_2)_2CH_3$), 2.25 (d, J = 5.7 Hz, 2 H, $CH_2CH(OEt)_2$), 3.45 and 3.63 (2 m, 4 H, $CH(OCH_2CH_3)_2$), 4.15 (q, J = 7.1 Hz, 4 H, $C(O)CH_2CH_3$), 4.51 (t, J = 5.7 Hz, 1 H, $CH_2CH(OEt)_2$); MS M^+ calcd. for $C_{17}H_{31}O_6$: 331.2121, found 331.2121, *m/e* 331 (0.5%, M^+), 287 (25%, $(M - OEt)^+$), 213 (26%), 173 (30%), 103 (100%, $(CH(OEt)_2)^+$), 75 (40%), 47 (30%).

5-(1-butyl)-5-(2-formylmethyl)barbituric acid (7). Sodium (1.43 g, 0.062 mol) was reacted with dry EtOH (50 ml) and ethyl 2-ethoxycarbonyl-2-(2,2-diethoxyethyl)hexanoate (4.14 g, 0.012 mol) and urea (0.75 g, 0.012 mol) were added. The mixture was heated overnight at 100 °C. After cooling, water was added and the solution was acidified with 5% aqueous HCl. The mixture was extracted with CH_2Cl_2 (50 x 3 ml), dried

(Na_2SO_4) and the solvent was evaporated to give a yellow oil which was crystallized from CH_2Cl_2 to give **7** as a white solid (1.5 g, 40%). Mp: 144-145 °C. ^1H NMR ($CDCl_3$) δ 0.85 (t, J = 7.1 Hz, 3 H, $(CH_2)_3CH_3$), 1.12 (t, J = 7.1 Hz, 6 H, OCH_2CH_3), 1.21(m, 4 H, $CH_2(CH_2)_2CH_3$), 1.91 (m, 2 H, $CH_2(CH_2)_2CH_3$), 2.42, (d, J = 5,9 Hz, 2 H, $CH_2CH(OEt)_2$), 3.37 and 5.59 (2 m, 4 H, OCH_2CH_3), 4.51 (t, J = 5.9 Hz, 1 H, $CH_2CH(OEt)_2$), 8.35 (bs, 2H, 2 NH); MS $(M - OEt)^+$ calcd. for $C_{12}H_{19}N_2O_4$: 255.1345, found 255.1344, *m/e* 255 (25%, $(M - OEt)^+$), 227 (10%), 184 (10%), 141 (30%), 116 (15%), 103 (100%, $(CH(OEt)_2)^+$), 75 (45%), 47 (55%). Anal. Calcd. for $C_{14}H_{24}N_2O_5$: C, 55.98; H, 8.05; N, 9.32. Found: C, 55.82; H, 8.09; N, 9.27.

The foregoing acetal derivative (1.4 g, 4.6 mmol) was dissolved in HCl (gaseous) saturated acetone (100 mL) . The mixture was stirred for 3 h at room temperature, the solvent was evaporated and the crude product was crystallized from CH_2Cl_2/hexanes to give 1.0 g (95 %) of **7**. Mp: 148-149 °C. ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 7.1 Hz, 3H, $(CH_2)_3CH_3$), 1.29 (m, 4 H, $CH_2(CH_2)_2CH_3$), 3.40 (s, 2 H, CH_2COH), 8.01 (bs, 2 H, NH), 9.58 (s, 1 H, CHO). Anal. Calcd. for $C_{10}H_{14}N_2O_4$: C, 53.09; H, 6.24; N, 12.38. Found: C, 53.17; H, 6.26; N, 12.33.

5-(1-butyl)-5-(2-hydroxycarbonylmethyl)barbituric acid (8a). To a solution of **7** (0.50 g, 2.2 mmol) in pyridine (20 ml) at 0 °C was added dropwise a solution containing potassium permanganate (0.27 g, 1.7 mmol), pyridine (10 ml) and water (10 mL). The reaction mixture was then stirred at room temperature for 24 h. The resultant suspension was filtered through celite which was then washed with pyridine. The filtrate was evaporated and the crude solid obtained was treated with 5% aqueous HCl and then extracted with Et_2O (3 x 50 mL). The organic extracts were combined, dried (Na_2SO_4) and evaporated to give a solid that was recrystallized from Et_2O/hexanes to afford 0.460 g (86 %) of **8a**. Mp: 199-200 °C. ^1H NMR ($CDCl_3$ + 5% DMSO-d_6) δ 0.85 (t, J = 6.6 Hz, 3 H, $(CH_2)_3CH_3$); 1.25 (m, 4 H, $CH_2(CH_2)_2CH_3$), 1.83 (m, 2 H, $CH_2(CH_2)_2CH_3$), 3.13 (bs, 2 H, CH_2COOH); 9,01 (bs, 2 H, NH); MS $(M - OH)^+$ calcd. for $C_{10}H_{13}N_2O_4$: 225.0875, found 225.0875, *m/e* 225 (1.5 %, $(M - OH)^+$), 213 (1%), 209 (1%), 186 (50%), 168 (90%), 142 (100%), 98 (20%), 85 (15%), 55 (20%). Anal. Calcd. for $C_{10}H_{14}N_2O_5$: C, 49.58; H,5.82; N, 11.56. Found: C, 49.44; H, 5.88; N, 11.47.

5-(1-butyl)-5-(2-(2,4-dinitrophenoxycarbonylmethyl))barbituric acid (8b). To a solution of **8a** (0.390 g, 1.61 mmol) in dry THF (20 ml) was added 2,4-dinitrophenol (0.296 g, 1.61 mmol), 1,3-dicyclohexyl-carbodiimide (0.332 g, 1.61 mmol) and 4-dimethylaminopyridine (10 mg, 0.082 mmol). The mixture was stirred overnight at room temperature under argon. The 1,3-dicyclohexylurea formed was removed by filtration and washed with THF. The filtrate was evaporated to give a yellow solid which was triturated with small portions of cold CH_2Cl_2. The product was purified by flash column chromatography (silica gel, CH_2Cl_2/Et_2O; 95/5) to give a pale yellow solid. Recrystallization from CH_2Cl_2/hexanes afforded 0.460 g (70 %) of **8b**. Mp 169-170 °C. ^1H NMR ($CDCl_3$) δ 0.91 (t, J = 7.1 Hz, 3 H, $(CH_2)_3CH_3$), 1.35 (m, 4 H, $CH_2(CH_2)_2CH_3$), 1.98 (m, 2 H, $CH_2(CH_2)_2CH_3$), 5.57 (s, 2 H, CH_2CO), 7.49 (d, J = 8.8 Hz, 1 H, H_3Ph, 7.85 (bs, 2 H, NH), 8.51 (dd, J = 8.8 and 2.7 Hz, 1 H, H_5Ph), 8.96 (d, J = 2.7 Hz, 1 H, H_6Ph); MS , *m/e* 225 (3%, M - 2,4-dinitrophenol), 184 (100%, 2,4-dinitrophenol), 168 (20%), 154 (30%), 138 (40%), 125 (20%), 110 (50 %), 91 (30%), 70 (50%), 63 (40%), 55 (45%). Anal. Calcd. for $C_{16}H_{16}N_4O_9$: C, 47.06; H, 3.95; N, 13.71. Found: C, 47.17; H, 4.00; N,13.72.

Kinetics. Solutions were prepared in distilled CH_2Cl_2 purged with argon. Slower reactions were followed on a Perkin-Elmer Lamba 5 or a Shimadzu UV-260 spectrophotometer. Faster reactions were followed on an Applied Photophysics Stopped-Flow spectrometer model SF.17 MW. The reaction temperature was maintained

at 25 ± 0.1 °C. Release of 2,4-dinitrophenol was followed through 5-6 half lives at 350 nm. Each kinetic run was initiated by injecting 20-40 μL of substrate (1 x 10^{-3} M in CH_2Cl_2) into the cuvette containing 2 mL of catalyst and 2,6-lutidine solution. Rate constants were obtained by non-linear regression analysis of the absorbance vs time curve (with the software package ENZFITTER by Leatherbarrow, R. J., Elsevier: Amsterdam, 1987). The errors in rate-constant measurements were always < 2%.

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