Design, Synthesis, Physicochemical Properties, and Evaluation of Novel Iron Chelators with Fluorescent Sensors

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The synthesis of a range of novel 3-hydroxypyridin-4-ones and 3-hydroxypyran-4-ones linked with different coumarin substituents is described. These compounds have been developed in order to provide a series of molecular probes for the quantification of intracellular labile iron pools. An evaluation of the effect of iron(III) on fluorescence intensity was undertaken. Chelation of iron(III) causes quenching of fluorescence. The relationship between iron(III) concentration and the extent of fluorescence quenching indicates that the metal is chelated in a complex with a metal-to-ligand stoichiometry of 1:3. The fluorescence of hydroxypyridinone compounds was found to be more efficiently quenched by iron(III) than were the hydroxypyranones. The metal-to-ligand stoichiometry at which maximum quenching is observed was found to depend on the site at which coumarin is attached. The efficiency of fluorescence quenching by iron(III) is markedly influenced by solvent polarity and pH. The permeability of two representative fluorescent chelators across human erythrocyte ghost membranes was investigated. The rate of permeability for a series of probes was found to be related to the corresponding ClogP values.

Iron is a ubiquitous metal in cells, it being present in the structure of many enzymes and proteins and therefore essential for cellular metabolism.[1] However, the presence of iron can also be detrimental, as it may facilitate the generation of highly reactive oxygen species. In the presence of molecular oxygen, labile iron is able to redox cycle between the two most stable oxidation states iron(II) and iron(III).[2] Hydroxyl radicals are highly reactive and capable of interacting with most types of biological molecule including sugars, lipids, proteins and nucleic acids, resulting in peroxidative tissue damage.[3] Since man lacks a physiological mechanism for excreting iron, iron homeostasis is largely achieved by the regulation of iron absorption.[4] Despite its high physiological and pathophysiological interest, only a few methods of detecting the chelatable iron pool in cells have been described.[5−14] These methods either require the destruction of the sample, lack the requisite sensitivity, or are not easily adaptable for high-throughput assays. Recently, fluorescent dyes in combination with digital fluorescence microscopy have become increasingly important for the detection of intracellular ions. In 1995, Cabantchik and co-workers introduced a fluorescent method for assessing the labile iron pool in cultured erythroleukemia K562 cells, based on the quenching of calcein fluorescence by metal ions.[15] Unfortunately, this method is not well suited for analogous measurements in hepatocytes, an important cell type in mammalian iron metabolism. The in situ calibration method is difficult to perform in a reliable and reproduceable manner. To quantitatively monitor the distribution of the labile iron pool in biological systems, the design of alternate iron-specific fluorescent probes is essential. Selective chelators are used to remove excess iron in a number of clinical situations and among a wide range of iron chelating compounds, 3-hydroxypyridin-4-ones (HPOs) are currently one of the main candidates for development of orally active iron chelators.[16] On the basis of selectivity and affinity, particularly considering the pFe^{3+} value, HPOs are the optimal bidentate ligands for the chelation of iron(III) over the pH range of 6.0−9.0. For this reason, we considered it appropriate to investigate the possibility of such molecules being converted into iron-specific fluorescent probes. In the present study, we have synthesized a series of coumarin-containing probes combined with either a hydroxypyridinone or hydroxy-pyranone moiety using a range of different links. The physicochemical properties of this series of compounds have been characterized. The permeability of representative probes across human erythrocyte membranes is reported.

Results

Chemistry. The synthetic route employed for the fluorescent moieties **4a**,**b** and **5a−f** is summarized in Schemes 1 and 2. 4-Substituted coumarins were synthesized by reaction of phenol derivatives with ethyl acetonedicarboxylate or ethyl 4-chloroacetoacetate by either use of Amberlyst-15 Cation-Exchange resin (method A) or zinc chloride (method B). With 3-substituted coumarins, piperidine and glacial acetic acid were used as catalyst in the reaction of substituted salicylaldehydes with diethyl malonate. The activated mercaptothiazoline coumarin derivatives **5a−f** were obtained by hydrolysis of the corresponding coumarin esters, followed by coupling with 2-mercaptothiazoline using dicyclohexylcarbodiimide (DCCI) and 4-(dimethylamino)pyridine (DMAP). The quaternary phosphonium

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Scheme 1. Synthesis of Compounds **4a,b** and **5a−d**
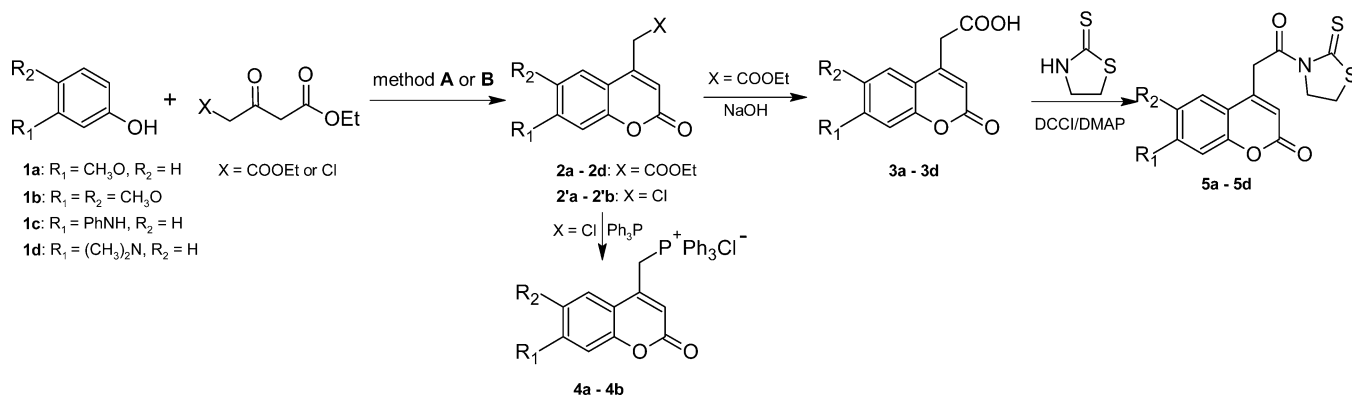
Scheme 2. Synthesis of Compounds **6e−f**
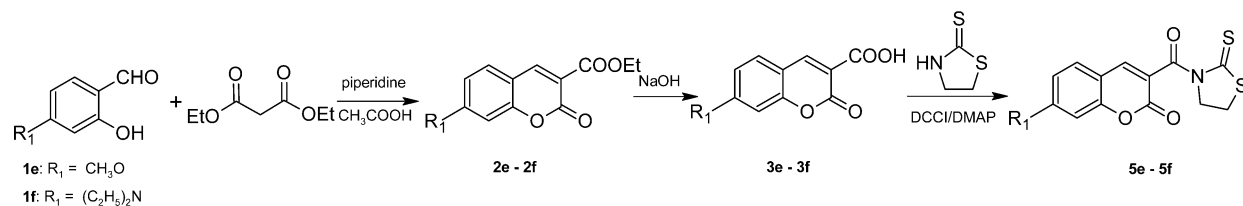
Scheme 3. Synthesis of Compounds **10a−h**
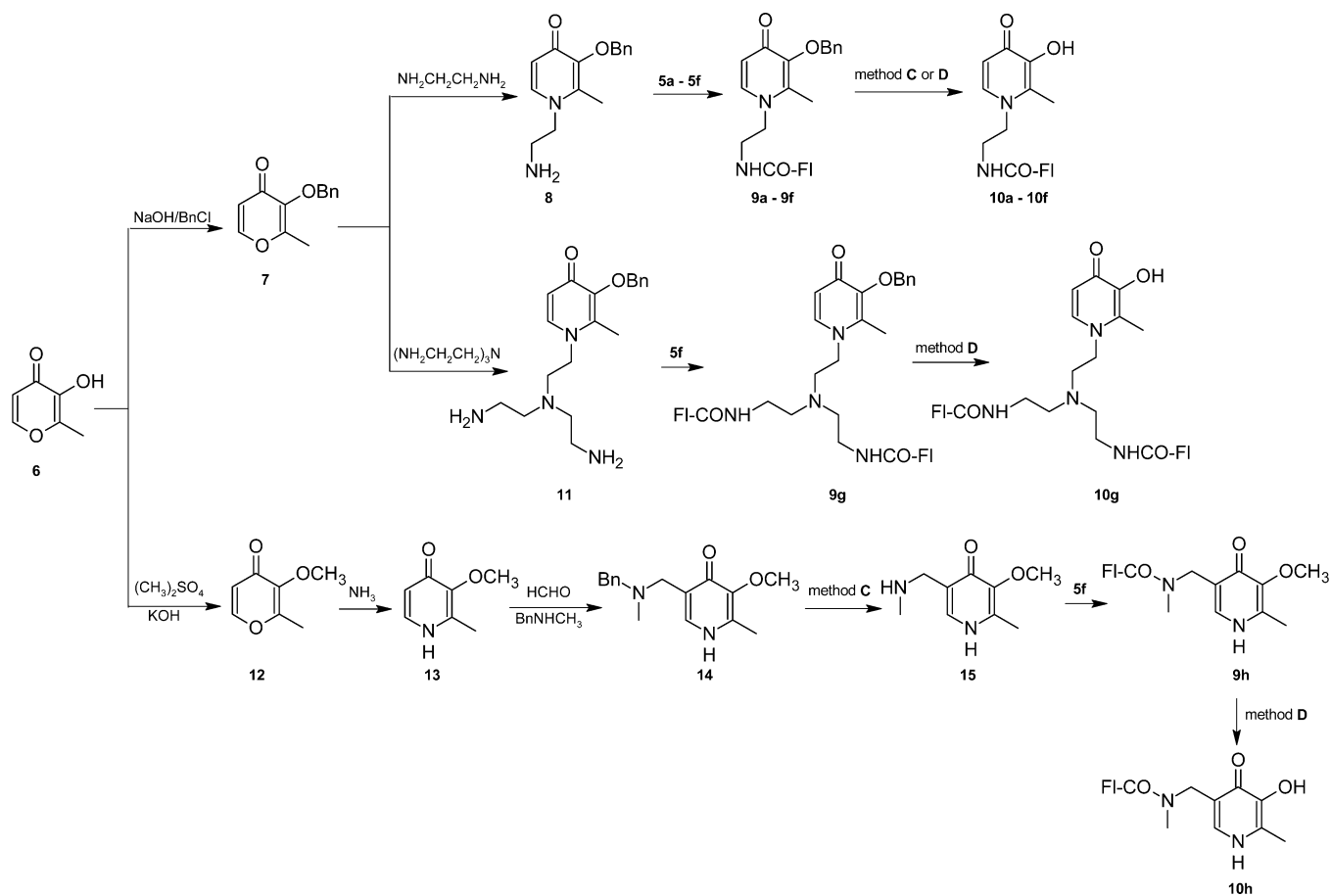
salts, **4a,b** were formed by addition of triphenylphosphine to the 4-chloromethylcoumarins **2′a,b**.

The general methodology adopted for the synthesis of 1-amido-3-hydroxypyridin-4-ones **10a−g** is summarized in Scheme 3. The 3-hydroxyl group was protected using the benzyl group, and the resulting protected maltol was then reacted with the corresponding amines to produce **8** and **11**, respectively, under mild conditions. The compounds **10a−g** were then synthesized by coupling the protected pyridinones **8** and **11** with the activated mercaptothiazoline coumarin derivative **5a−f**, followed by catalytic hydrogenation (method C) or BCl_3 (method D) to remove the protecting benzyl group. The 5-amido-hydroxypyridinone **10h** can be further

modified using the methodology illustrated in Scheme 3. Methylation of maltol was achieved using dimethyl sulfate under basic conditions, 2-methyl-3-methoxy-4(1*H*)-pyridinone was obtained by the reaction of the protected maltol with ammonia. 5-Methylaminomethylpyridinone **15** was then prepared by the reaction of compound **13** with formaldehyde and benzylmethylamine under Mannich reaction conditions, followed by hydrogenation to remove the benzyl group.

The synthetic route for the synthesis of 2- or/and 6-aryl-substituted pyranones and pyridinones (**10i**−**w**) is summarized in Scheme 4. 6-Formyl-3-benzyloxypyran-4(1*H*)-one **18** was obtained by the benzylation of kojic acid followed by selective oxidation with sulfur trioxide pyridine complex in combination with dimethyl sulfoxide. The double bond was formed by Wittig reaction of aldehyde **18** with phosphonium salt **4a,b**. The deprotection of compound **9j** by two different methods (method c and method d) afforded two different products **10j** and **10'j**.

The conversion of 6-hydroxymethyl-3-benzyloxypyran-4(1*H*)-one **17**, 2-hydroxymethyl-6-methyl-3-benzyloxypyran-4(1*H*)-one **27a**, or 2-hydroxymethyl-6-methyl-3-benzyloxypyridin-4(1*H*)-one **27b** to the corresponding phthalimido intermediates, respectively, **19**, **28a**, or **28b**, was accomplished in good yield using phthalimide, triphenylphosphine, and diethyl azodicarboxylate (DEAD). The reaction is believed to proceed through the formation of a quaternary phosphonium salt by addition of triphenylphosphine to DEAD, which reacts in situ with **17**, **27a**, or **27b** to form an alkoxyphosphonium salt.[17] The phosphonium moiety was then displaced by the phthalimido anion to furnish the desired phthalimido derivatives **19**, **28a**, or **28b** respectively, which were subsequently reacted with hydrazine to afford the corresponding amine derivatives **20**, **29a**, or **29b**. The method of synthesis of the phthalimido intermediate from **21** with phthalimide according to the above procedure only produced low yields (less than 20%) Therefore, a different synthetic method for the amino compound **23** was developed where the hydroxyl group of **21** was converted to a chloride with thioyl chloride, followed by the addition of ammonia. An improved yield of over 80% was obtained. The direct conversion of the benzylated 6-hydroxymethylpyranone **17** to the corresponding *N*-methylpyridinone analogue **21** was successful when water was used as a solvent. However, the yield of the direct conversion of the benzylated 2-hydroxmethylpyranone **27a** to the corresponding *N*-methylpyridinone analogue **27b** was lower than that of the conversion of **17** to **21**. As a result of this finding, the 2-hydroxyl function of **27a** was protected using 3,4-dihydro-2*H*-pyran before reacting with methylamine. The pyran protecting group was then selectively removed under mild acidic conditions without deprotection of the benzyl group, resulting in the desired protected 2-hydroxymethylpyridinone **27b** in a much improved yield.

Molecular Structures of Iron Probes. The structures of a range of 3-hydroxypyridin-4-ones and 3-hydroxypyran-4-ones linked with different coumarin substituents are presented in Table 1. The coumarin substituent was linked to 3-hydroxypyridin-4-one at the 1-position (**10a**−**f**), 2-position (**10s**−**v**), 5-position (**10h**),

and 6-position (**10n**, **10o**). Similarly, the coumarin substituent was linked to the 3-hydroxypyran-4-one nucleus at the 2-position (**10p**−**r**) and 6-position (**10i**−**m**). In addition, one probe (**10g**) was prepared containing two fluorescent groups linked to 3-hydroxypyridin-4-one.

Six types of coumarin (Fl$_a$−Fl$_f$, see Table 1) were selected as fluorescence units in this molecular series. These fluorescent moieties were linked to the pyridinone and pyranone moieties utilizing amido and amino functions (**10n**), carbon−carbon single bonds (**10'j**) and carbon−carbon double bonds (**10i** and **10j**).
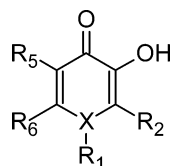
Fluorescent Properties. Coumarin itself is not fluorescent; however, the addition of various electron donating substituents at the 6- and 7-positions result in a red shift and an enhanced fluorescence intensity.[18] The emission wavelength (Em) of the methoxycoumarin derivatives lies in the 380−423 nm range (Table 2), dimethoxycoumarin possessing the highest Em, namely 423 nm. When the dimethylamino group is introduced at the 7-position of coumarin, an increase in the Em value was observed (473 nm). The effect was similar with the introduction of the diethylamino group (470 nm). However, when the methoxy group was substituted by the phenylamino moiety (**10c**), fluorescence was abolished. When the iron chelator and fluorescent probe components are conjugated (for example: **10i** and **10j**), the fluorescence is abolished. A similar effect occurs when the two moieties are connected by an amino link (**10n**). The probable reason for these latter observations is that the double bond or the amino link between the coumarin and the chelator moiety changes the electron distribution in the coumarin ring. A similar phenomenon occurs with the phenyl group of probe **10c**. In contrast, the presence of an amido link between the two moieties does not appreciably influence the fluorescence properties, probably because the amido group is able to attract electrons from the electron donor group at the 7-position of coumarin.

Not surprisingly, the different chelator moieties did not appreciably influence the fluorescent properties of the free ligand as judged by excitation wavelength, emission wavelength and extinction coefficient (see for example: **10f**, **10h**, **10o**, and **10v**). However, the quantum yield was found to be larger for the 3-hydroxypyran-4-ones when compared with the 3-hydroxypyridin-4-ones (**10a** to **10p**, **10b** to **10q**, **10e** to **10r**). The probable reason for the decrease of the quantum yield is that the 3-hydroxypyridin-4-one ring exists as a highly polarized aromatic structure in which nitrogen is positively charged and oxygen at the 4-position of 3-hydroxypyridin-4-one is negatively charged.[19]

Surprisingly, the fluorescent intensity of probe **10g** which contains two 7-diethylaminocoumarin groups is weak in water, indeed virtually undetectable. Bond-rotation in the molecule induced by the hydrophilic solvent leads to an overlap of the two fluorescent probes, which in turn causes self-quenching. In an attempt to avoid this type of interaction, the molecule was investigated in methanol, whereupon the fluorescent intensity increased although not reaching twice that of probe **10f**.

Fluorescence Quenching. The fluorescent emission intensity of the pyridinone fluorescent probes was found

Scheme 4. Synthesis of Compounds **10i–v** and **10j'**
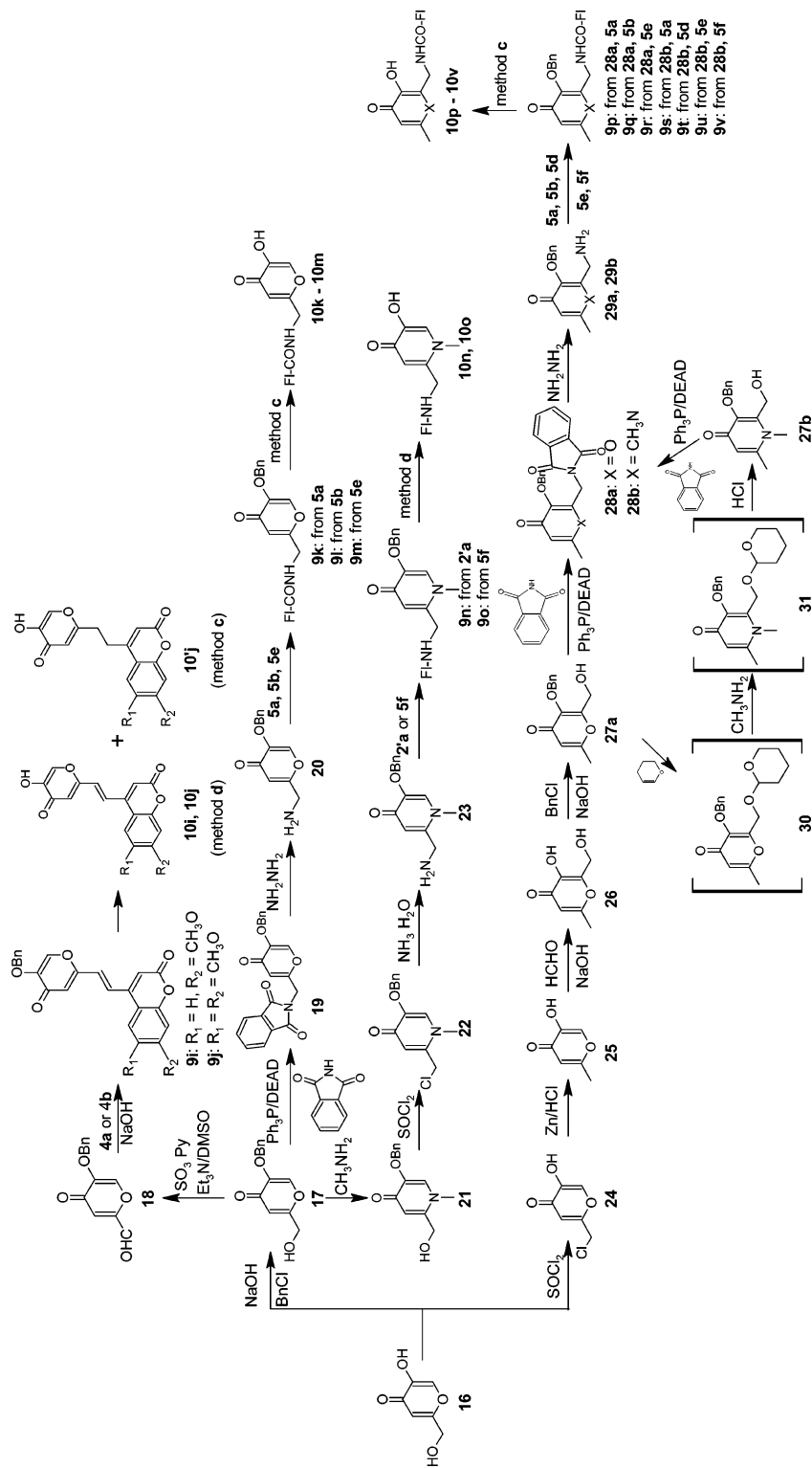
Table 1. Chemical Structure of Fluorescent Probes

compd	X	$R_1{}^a$	R_2	R_5	R_6
10a	N	$Fl_aCH_2CONHCH_2CH_2$	CH_3	H	H
10b	N	$Fl_bCH_2CONHCH_2CH_2$	CH_3	H	H
10c	N	$Fl_cCH_2CONHCH_2CH_2$	CH_3	H	H
10d	N	$Fl_dCH_2CONHCH_2CH_2$	CH_3	H	H
10e	N	$Fl_eCONHCH_2CH_2$	CH_3	H	H
10f	N	$Fl_fCONHCH_2CH_2$	CH_3	H	H
10g	N	$(Fl_fCONHCH_2CH_2)_2NCH_2CH_2$	CH_3	H	H
10h	N	H	CH_3	$Fl_fCON(CH_3)CH_2$	H
10i	O	-	H	H	$Fl_aCH=CH$
10j	O	-	H	H	$Fl_bCH=CH$
10j′	O	-	H	H	$Fl_bCH_2CH_2$
10k	O	-	H	H	$Fl_aCH_2CONHCH_2$
10l	O	-	H	H	$Fl_bCH_2CONHCH_2$
10m	O	-	H	H	$Fl_eCONHCH_2$
10n	N	CH_3	H	H	$Fl_aCH_2NHCH_2$
10o	N	CH_3	H	H	$Fl_fCONHCH_2$
10p	O	-	$Fl_aCH_2CONHCH_2$	H	CH_3
10q	O	-	$Fl_bCH_2CONHCH_2$	H	CH_3
10r	O	-	$Fl_eCONHCH_2$	H	CH_3
10s	N	CH_3	$Fl_aCH_2CONHCH_2$	H	CH_3
10t	N	CH_3	$Fl_dCH_2CONHCH_2$	H	CH_3
10u	N	CH_3	$Fl_eCONHCH_2$	H	CH_3
10v	N	CH_3	$Fl_fCONHCH_2$	H	CH_3

^{a}
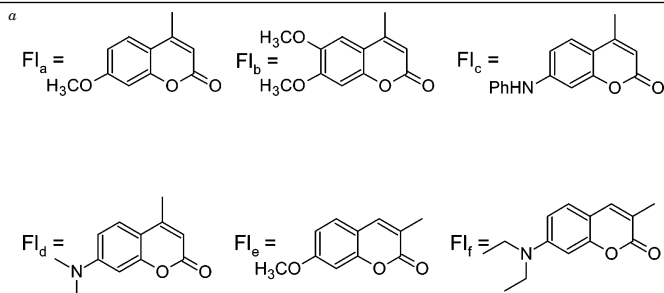
to be sensitive to the presence of metal ions, and a clear quenching effect was observed on the addition of ferric ions. In contrast, the iron(III)-induced quenching of the fluorescent emission intensity of the pyranone fluorescent probes was found to be comparatively moderate. The major reason for this difference is that the affinity constants (β_3) of the pyridinone fluorescent probes for iron(III) are approximately 10^4-fold greater than those of the pyranone probes.[20] The specific quenching ratio of these fluorescent probes is shown in Table 2. The quenching ratios of pyridinone fluorescent probes fall between 46.4% (**10u**) and 95.1% (**10o**) and those of the pyranone fluorescent probes between 0% (**10′j**) and 46.4% (**10p**). A clear example for the difference of quenching ratios between pyridinones and pyranones is that the quenching ratio of the probe **10o**, where the fluorescent moiety is linked to the pyridinone site at the 6-position, is 95.1%; whereas, the quenching ratios of the probes **10k**, **10l**, and **10m**, where the fluorescent moiety is linked at the same position on the pyranone nucleus, are 18.5%, 15.9%, and 27.5%, respectively.

The mechanism of fluorescence quenching was assessed for probe **10a**. The relationship between iron concentration and fluorescence quenching was determined, and the results are plotted in a Stern−Volmer plot (Figure 1). The relationship between F_0/F vs [Fe] is nonlinear, indicating that the mechanism of quenching is static rather than dynamic.[46] This is consistent with the action of the probe as an iron chelator, the result of which causes loss of fluorescence properties.

Prediction of Intracellular Distribution. By virtue of Lipinski's rule-of-five, four parameters namely molecular weight, LogP, the number of H-bond donors, and the number of H-bond acceptors can be used to predict the likelihood of a molecule crossing biological membranes by simple diffusion.[21] The fluorescent probes (**10a−f**, **10h−v**) are relatively small in size, their molecular weights being less than 500; they also possess less than 5 H-bond donors and 10 H-bond acceptors. As the ClogP values for this group of molecules are all less than 5, it would appear likely that the entire group will readily penetrate membranes and are therefore favorable for efficient intracellular distribution. Exceptionally the molecular weight of probe **10g** is rather more than 500, the number of H-bond acceptors is close to 10, and the ClogP value is close to 5. Thus **10g** is not predicted to efficiently permeate cell membranes.

Permeability across Human Erythrocyte Membranes. The permeability of two representative fluorescent chelators, **10a** and **10v**, differing in ClogP values

Table 2. Physiochemical Properties of Probes[a]

|	Ex, λ (nm)	Em, λ (nm)	ϵ,[b] $\times 10^3$ M^{-1} cm^{-1}	Φ_F[c]	quenching[d] (Fe^{3+}) (%)	MW[e]	no. of H donors	no. of H acceptors	ClogP
10a	325	380	12.2	0.04	80.6	420	2	5	−0.48
10b	349	423	10.4	0.04	59.9	450	2	6	−0.80
10c	380	ND[f]	- -	- -	- -	481	3	5	1.56
10d	387	473	6.8	0.11	50.6	433	2	5	−0.14
10e	351	397	25.2	0.02	49.4	406	2	5	−0.02
10f	431	468	31.7	0.01	93.9	447	2	5	1.55
10g	412	ND	- -	- -	- -	813	3	9	4.43
10h	416	477	24.5	0.02	76.5	447	2	5	0.43
10i	314	ND	- -	- -	- -	312	1	4	1.55
10j	314	ND	- -	- -	- -	342	1	5	1.23
10j′	342	412	7.6	0.30	0	344	1	5	1.38
10k	324	382	12.3	0.14	18.5	357	2	5	−0.17
10l	346	423	10.3	0.14	15.9	387	2	6	−0.49
10m	349	397	22.7	0.51	27.5	343	2	5	0.39
10n	322	ND	- -	- -	- -	415	2	5	−1.22
10o	432	470	38	0.01	95.1	433	2	5	0.69
10p	325	380	12.7	0.18	46.4	371	2	5	0.28
10q	345	420	11.4	0.24	32.8	401	2	6	−0.04
10r	349	397	24.9	0.12	9.8	357	2	5	0.84
10s	325	380	11.4	0.06	65.4	420	2	5	−0.72
10t	387	474	14.3	0.11	77.3	433	2	5	−0.39
10u	349	397	20.6	0.01	46.4	406	2	5	−0.17
10v	431	470	42.1	0.01	79.2	447	2	5	1.14

[a] Conditions of analysis. [Fe^{3+}] 0.5 μM; [probe] 3–15 μM; MOPS buffer pH 7.4. [b] Molar extinction coefficient. [c] Quantum yield. [d] Quenching of fluorescence intensity (%) (metal-to-ligand molar ratio 1:3). [e] Molecular weight. [f] No detectable fluorescence.

**Table 3.** Permeability Parameters for Fluorescent Probes across Human Erythrocyte Ghost Membranes

	10a				**10v**			
	A_1	t_1	A_2	t_2	A_1	t_1	A_2	t_2
− DTPA	37.4 ± 1.1	18.4 ± 0.9	87.9 ± 0.4	221.7 ± 1.3	124.2 ± 0.9	38.0 ± 0.5	269.1 ± 1.0	212.3 ± 0.6
+ DTPA	42.4 ± 1.0	84.7 ± 2.7	-	-	137.5 ± 3.6	61.0 ± 2.2	-	-
ClogP	−0.48				1.14			
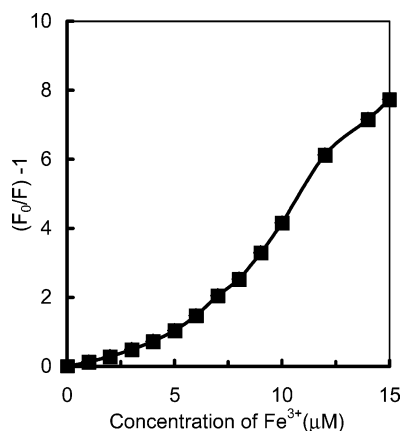
Figure 1. Stern−Volmer plot of fluorescence quenching of 15 μM **10a** by Fe(III) in MOPS buffer (25 mM), pH 7.4.

(−0.45 and 1.14, respectively) across human erythrocyte ghost membranes was determined (Figure 2). Red cell ghosts were resealed in the presence of iron(II) ammonium sulfate and ascorbic acid and washed to remove untrapped iron. The fluorescence intensity of the probe was monitored during a 30 min incubation at 20 °C with washed erythrocyte ghost suspensions in the presence and absence of the impermeant nonfluorescent iron chelator, DTPA. Changes in fluorescence intensity as a function of incubation time are presented in Figure 2. The fluorescence was quenched to a greater extent in the absence of DTPA because under these conditions iron not only quenched the chelator that permeates into the resealed ghosts, but also iron leaked from the ghosts and quenched the fluorescence of the probe present in
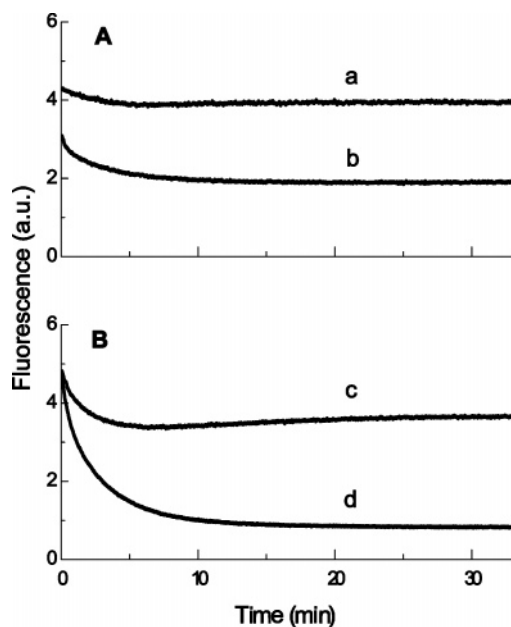
Figure 2. Fluorescence intensity of A, 15 μM **10a** (λ_{ex} 325; λ_{em} 380); B, 6 μM **10v** (λ_{ex} 431; λ_{em} 470) recorded during incubation at 20 °C in the presence of suspensions of washed human erythrocyte ghosts resealed in the presence of 100 μM Fe^{2+}, 1 mM ascorbic acid. The suspension medium also contained 1 mM ascorbic acid plus 0.1 mM DTPA (a, c) and 1 mM ascorbic acid (b, d).

the suspension medium. The two processes are consistent with a fit of the respective data to a double exponential decay function as shown in Table 3. With incubations performed in the presence of excess DTPA
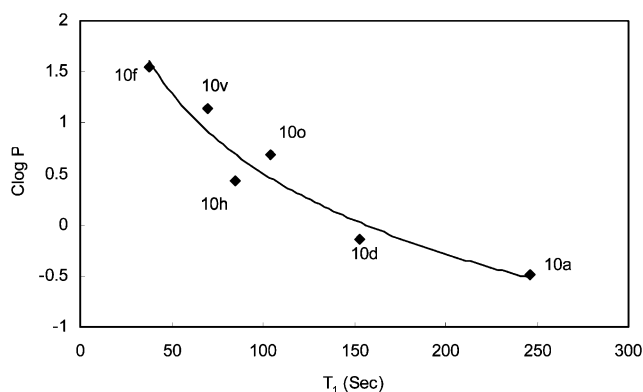
Figure 3. Relationship between permeability across the human erythrocyte ghost membrane and ClogP value of **10a** (λ_{ex} 325; λ_{em} 380); **10d** (λ_{ex} 387; λ_{em} 473), **10f** (λ_{ex} 431; λ_{em} 468), **10h** (λ_{ex} 416; λ_{em} 477), **10o** (λ_{ex} 432; λ_{em} 470), **10v** (λ_{ex} 431; λ_{em} 470) in the presence of 100 μM Fe^{2+}, 1 mM ascorbic acid and 0.1 mM DTPA. All probes were investigated at 15 μM.
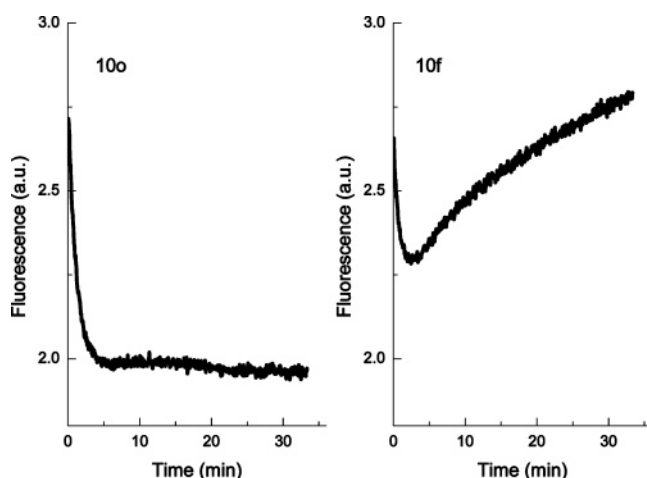
Figure 4. Fluorescence intensity of **10o** (15 μM, λ_{ex} 432; λ_{em} 470) and **10f** (15 μM, λ_{ex} 431; λ_{em} 468) recorded during incubation at 20 °C in the presence of suspensions of washed human erythrocyte ghosts resealed in the presence of 100 μM Fe^{2+}, 1 mM ascorbic acid. The suspension medium also contained 1 mM ascorbic acid and 0.1 mM DTPA.

in the suspending medium, fluorescence quenching due to the leakage of iron from the ghost membranes is avoided. As expected, these data fit single-exponential decay functions, representing permeation of the fluorescent chelator into the resealed ghosts. A slight increase in fluorescence intensity observed during the incubation is due to the efflux of the fluorescent chelator from the resealed ghosts into the suspension medium. The comparison of the relaxation times and related weighting functions of six fluorescent chelators demonstrated a direct relationship between permeability across the erythrocyte ghost membrane and ClogP values (Figure 3).

The behavior of two representative probes (**10o** and **10f**) with the ClogP values 0.69 and 1.55, respectively, in the presence of human erythrocyte ghost membranes was recorded over a 30 min period (Figure 4). The fluorescence associated with **10o** was found to be stable in the presence of human erythrocyte ghosts. In contrast with **10f** the fluorescence failed to stabilize. This difference in behavior is readily explained by the estimated LogD values of the corresponding 3:1 iron(III) complexes of the two molecules which are calculated [22] as ap-

proximately 1 and 3, respectively. Thus the estimated *D* value of compound **10f** is 100 times larger than that of compound **10o**. Clearly the iron complex of **10f** by virtue of its enhanced lipophilicity penetrates the ghost membrane more readily and therefore effluxes from the cell (Figure 4) in marked contrast to the more hydrophilic analogue, **10o**.

Discussion

It has previously been established that the novel fluorescent probes described in this paper are more selective sensors of iron(III) than calcein.[23] Furthermore, calcein suffers from a number of limitations, namely it is not membrane permeable (and therefore, even with the use of ester proprobe analogues, cannot be used to monitor iron levels in intracellular organelle matrixes), it possesses several different chelation modes with iron(III), [24] and it is a substrate for multiple drug resistance (MDR) receptor proteins. The probes described in this work are based either on 3-hydroxypyridin-4-ones or 3-hydroxypyran-4-ones, and so they possess similar iron(III) binding properties to those of compounds **10a** (CP600) and **10p** (CP610).[23] The high affinity of these well characterized chelators of iron(III) is known to facilitate the autoxidation of iron(II) under aerobic conditions.[20] Thus, the novel probes are predicted to be suitable to monitor combined levels of iron(II) and iron(III).

The probes bind iron optimally in a 3:1 molar ratio; thus with **10a** the free ligand has a molecular weight of 421, whereas the resulting iron complex has the much higher molecular weight of 1,316. Thus although the probes are able to enter cells by simple diffusion, once coordinated to iron they efflux much more slowly. Thus in principle it should be possible to monitor intracellular iron pools, if the chelation by iron leads to a change in fluorescent properties.

In this investigation we have demonstrated that the bidentate hydroxypyridinone-based probes are associated with a greater iron-induced fluorescence quenching effect than that of their hydroxypyranone analogues. Moreover, the hydroxypyridinone probes are generally more water soluble than their hydroxypyranone counterparts. The probable reason for this latter observation is that the charged mesomeric contribution associated with the hydroxypyridinone molecules makes a greater contribution than with the equivalent hydroxypyranone mesomer. Potentially useful HPO probes (**10a, 10d, 10h, 10o, 10v**, and **10f**) were selected on the basis of iron(III)-induced fluorescence quenching (Table 2). They possess a range of ClogP values from −0.48 to +1.55. To check for their suitability for cell studies, we monitored the ability of these selected probes to penetrate erythrocyte ghost membranes. Probes with ClogP values >0 were found to penetrate the membrane efficiently (Figure 3), and we believe that these probes have potential for imaging intracellular iron pools. Compound **10o** would appear to be optimal for monitoring intracellular iron pools, as it has the largest fluorescence quenching and weighting values of all the compounds presented in Table 2 and when dissolved in water is extremely sensitive to stoichiometric binding with iron(III). The formation of the relatively hydrophilic 3:1 iron complex associated with **10o** within the erythrocyte leads to a

stable fluorescence signal. Clearly the intracellular levels of the iron complex remain constant for at least 30 min (Figure 4).

Compound **10o** possesses a fluorescence emission maximum wavelength of 470 nm (Table 2). This excellent signaling property of fluorescence is predicted to facilitate the potential of probe **10o** for application in determining intracellular labile iron levels by confocal microscopy techniques. Studies are currently in progress with a range of 3-hydroxypyridin-4-one-based probes in both hepatocytes and leucocytes.

Experimental Section

General Procedure. Methyl maltol (**6**) was purchased from Cultor Food Science. Kojic acid (**16**) was purchased from Fluka. Sepharose 2B was obtained from Pharmacia Ltd. All other chemicals were obtained from Sigma-Aldrich. Melting points were determined using an Electrothermal IA 9100 Digital Melting Point Apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Bruker (360 MHz) NMR spectrometer. Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). Mass spectra (FAB) analyses were carried out by the Mass Spectrometry Facility, School of Health and Life Science, 150 Stamford St., London SE1 9NN, UK.

Ethyl (7-methoxy-2-oxo-2H-chromen-4-yl)acetate (2a): Method A. To a solution of 3-methoxyphenol (**1a**) (6.2 g, 0.05 mol) and ethyl acetonedicarboxylate (11.1 g, 0.055 mol) in toluene (200 mL) was added Amberlyst-15 cation-exchange resin (3 g). The reaction mixture was heated under reflux with stirring using a Dean–Stark apparatus until no more water was produced (about 10 h). The hot reaction mixture was filtered to remove the catalyst, and upon cooling a yellow precipitate was obtained (if no precipitate occurred, evaporation of the solvent is necessary). Recrystallization from ethanol produced the coumarin as pale yellow powder solid (8.1 g, 62%): mp 100–102 °C (lit. value[25] 101–102 °C);^{1}H NMR (DMSO-d_6) δ: 1.30 (t, $J = 7.2$ Hz, 3H), 3.70 (s, 2H), 3.75 (s, 3H), 4.20 (q, $J = 7.2$ Hz, 2H), 6.10 (s, 1H), 6.70–6.90 (m, 2H), 7.42 (d, $J = 9.6$ Hz, 1H).

Analogous reactions of **1a** or **1b** with ethyl acetonedicarboxylate or ethyl 4-chloroacetoacetate gave compounds **2b**, **2′a**, and **2′b**.

Ethyl (6,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetate (2b): (9.9 g, 68%); mp 140–141 °C; ^{1}H NMR (DMSO-d_6) δ: 1.35 (t, $J = 7.2$ Hz, 3H), 3.75 (s, 3H), 3.81 (s, 2H), 3.87 (s, 3H), 4.25 (q, $J = 7.2$ Hz, 2H), 6.05 (s, 1H), 6.80 (s, 1H), 6.88 (s, 1H).

4-(Chloromethyl)-7-methoxy-2H-chromen-2-one (2′a): (6.7 g, 60%); mp 194–195 °C (lit. value[26] 195 °C); ^{1}H NMR (DMSO-d_6) δ: 3.84 (s, 3H), 4.55 (s, 2H), 6.30 (s, 1H), 6.72 (d, $J = 2.0$ Hz, 1H), 6.80 (dd, $J = 2.2, 8.4$ Hz, 1H), 7.47 (d, $J = 8.4$ Hz, 1H).

4-(Chloromethyl)-6,7-dimethoxy-2H-chromen-2-one (2′b): (7.8 g, 61%); mp 204.5–205.5 °C (lit. value[27] 194–196 °C); ^{1}H NMR (DMSO-d_6) δ: 3.80 (s, 3H), 3.85 (s, 3H), 5.01 (s, 2H), 6.45 (s, 1H), 7.08 (s, 1H), 7.22 (s, 1H).

Ethyl (7-Anilino-2-oxo-2H-chromen-4-yl)acetate (2c): Method B. To a solution of 3-anilinophenol (9.3 g, 0.05 mol) and ethyl acetonedicarboxylate (11.1 g, 0.055 mol) in anhydrous EtOH (30 mL) was added anhydrous ZnCl$_2$ (8.2 g, 0.06 mol), and the reaction mixture was refluxed overnight. After cooling, the reaction mixture was poured into ice–water under stirring. The resulting separated dark oil slowly solidified on being left in contact with cold EtOH. Recrystallization from EtOH gave needles (6.8 g, 42%): mp 148–149.5 °C (lit. value[28] 150–151 °C); ^{1}H NMR (CDCl$_3$) δ: 1.30 (t, $J = 7.2$ Hz, 3H), 3.69 (s, 2H), 4.20 (q, $J = 7.2$ Hz, 2H), 6.10 (s, 1H), 6.80–7.50 (m, 8H).

An analogous procedure starting with **1d** gave **ethyl [7-(dimethylamino)-2-oxo-2H-chromen-4-yl]acetate (2d):** (4.1 g, 30%); mp 129–131 °C (lit. value[29] 131–132 °C);

^{1}H NMR (CDCl$_3$) δ: 1.22 (t, $J = 7.2$ Hz, 3H), 3.02 (s, 6H), 3.61 (s, 2H), 4.12 (q, $J = 7.2$ Hz, 2H), 5.94 (s, 1H), 6.35–6.60 (m, 2H), 7.28 (d, $J = 8.4$ Hz, 1H).

Ethyl 7-Methoxy-2-oxo-2H-chromene-3-carboxylate (2e). A solution of 4-methoxysalicylaldehyde (7.6 g, 0.05 mol) and diethyl malonate (9.6 g, 0.06 mol) in EtOH (60 mL) was treated with piperidine (0.5 mL) and glacial acetic acid (2 drops) and refluxed for 3 h. To the reaction mixture was added 100 mL of H$_2$O, and the mixture was cooled to 0 °C. The crystalline solid was filtered and washed by 50% cold ethanol (20 mL). Recrystallization from 50% EtOH gave colorless crystals (8.8 g, 71%); mp 135–137 °C (lit. value[30] 117 °C); ^{1}H NMR (CDCl$_3$) δ: 1.39 (t, $J = 7.2$ Hz, 3H), 3.84 (s, 3H), 4.32 (q, $J = 7.2$ Hz, 2H), 6.70 (d, $J = 2.3$ Hz, 1H), 6.84 (dd, $J = 2.3, 8.4$ Hz, 1H), 7.42 (d, $J = 8.4$ Hz, 1H), 8.37 (s, 1H).

An analogous procedure starting with **1f** gave **ethyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate (2f)** as a yellow crystalline solid : (7.1 g, 49%); mp 75–76 °C (lit. value[31] 77–78 °C); ^{1}H NMR (CDCl$_3$) δ: 1.22 (t, $J = 7.2$ Hz, 6H), 1.37 (t, $J = 7.2$ Hz, 3H), 3.42 (q, $J = 7.2$ Hz, 4H), 4.34 (q, $J = 7.2$ Hz, 2H), 6.40 (d, $J = 2.2$ Hz, 1H), 6.56 (dd, $J = 2.2, 9.0$ Hz, 1H), 7.30 (d, $J = 9.0$ Hz, 1H), 8.45 (s, 1H).

(7-Methoxy-2-oxo-2H-chromen-4-yl)acetic Acid (3a). To a solution of ethyl (7-methoxy-2-oxo-2H-chromen-4-yl)acetate (**2a**) (2.62 g, 10 mmol) in EtOH (50 mL) was added 250 mL of 0.5% NaOH, and the mixture was heated under reflux for 2 h. Acidification to pH 2 using concentrated hydrochloric acid and cooling to 0 °C gave a crystalline deposit (1.87 g, 80%): mp 166–167 °C (lit. value[32] 167–170 °C); ^{1}H NMR (DMSO-d_6) δ: 3.70 (s, 2H), 3.75 (s, 3H), 6.10 (s, 1H), 6.65–6.88 (m, 2H), 7.42 (d, $J = 9.6$ Hz, 1H).

Analogous procedures starting with **2b–d** gave compounds **3b–d**.

(6,7-Dimethoxy-2-oxo-2H-chromen-4-yl)acetic acid (3b): (2.0 g, 78%); mp 174.5–175 °C; ^{1}H NMR (DMSO-d_6) δ: 3.76 (s, 3H), 3.81 (s, 2H), 3.85 (s, 3H), 6.04 (s, 1H), 6.80 (s, 1H), 6.88 (s, 1H).

(7-Anilino-2-oxo-2H-chromen-4-yl)acetic acid (3c): (2.21 g, 75%); mp 183.5–184.5 °C (lit. value[28] 186–189 °C); ^{1}H NMR (DMSO-d_6) δ: 3.64 (s, 2H), 4.20 (br s, 1H), 5.93 (s, 1H), 6.65–7.40 (m, 8H).

(7-Dimethylamino-2-oxo-2H-chromen-4-yl)acetic acid (3d): (2.00 g, 81%); mp 163.5–164.5 °C (lit. value[29] 166–167 °C); ^{1}H NMR (DMSO-d_6) δ: 3.00 (s, 6H), 3.71 (s, 2H), 5.94 (s, 1H), 6.40–6.75 (m, 2H), 7.40 (d, $J = 10.2$ Hz, 1H).

7-Methoxy-2-oxo-2H-chromen-3-carboxylic Acid (3e). To a solution of ethyl 7-methoxy-2-oxo-2H-chromene-3-carboxylate (**2e**) (2.48 g, 10 mmol) in EtOH (15 mL) was added 15 mL of 10% NaOH and heated under reflux for 15 min. Acidification to pH 2 using concentrated hydrochloric acid and cooling to 0 °C gave a crystalline deposit (1.87 g, 85%): mp 191.5–192.5 °C (lit. value[33] 176–177 °C); ^{1}H NMR (DMSO-d_6) δ: 3.80 (s, 3H), 6.72 (d, $J = 2.3$ Hz, 1H), 6.84 (dd, $J = 2.3, 8.4$ Hz, 1H), 7.40 (d, $J = 8.4$ Hz, 1H), 8.38 (s, 1H).

An analogous procedure starting with **2f** gave **7-diethylamino-2-oxo-2H-chromen-3-carboxylic acid (3f):** (2.14 g, 82%); mp 224–225.5 °C (lit. value[34] 215–217 °C); ^{1}H NMR (DMSO-d_6) δ: 1.22 (t, $J = 7.2$ Hz, 6H), 3.42 (q, $J = 7.2$ Hz, 4H), 6.40 (d, $J = 2.2$ Hz, 1H), 6.55 (dd, $J = 2.2, 9.0$ Hz, 1H), 7.30 (d, $J = 9.0$ Hz, 1H), 8.49 (s, 1H).

2-Methyl-3-benzyloxypyran-4(1H)-one (7). To a solution of methyl maltol **6** (63 g, 0.5 mol) in methanol (500 mL) was added sodium hydroxide (22 g, 0.55 mol) dissolved in water (50 mL), and the mixture was heated to reflux. Benzyl chloride (70 g, 0.55 mol) was added dropwise over 30 min, and the resulting mixture was refluxed overnight. After removal of solvent by rotary evaporation, the residue was mixed with water (200 mL) and extracted with dichloromethane (3 × 150 mL). The combined extracts were washed with 5% aqueous sodium hydroxide (2 × 200 mL) followed by water (200 mL). The organic fraction was then dried over anhydrous sodium sulfate, filtered, and rotary evaporated to yield an orange oil which solidified on cooling. Recrystallization from diethyl ether gave the pure product **7** as colorless needles (87.5 g, 81%); mp

51−52 °C (lit. value[35] 53−55 °C); ^{1}H NMR (CDCl$_3$) δ: 2.12 (s, 3H), 5.11 (s, 2H), 6.25 (d, J = 6 Hz, 1H), 7.28 (s, 5H), 7.47 (d, J = 6 Hz, 1H).

1-(2-Aminoethyl)-3-(benzyloxy)-2-methylpyridin-4(1*H*)-one (8). To a solution of **7** (2.16 g, 10 mmol) in water (100 mL) was added ethylenediamine (1.2 g, 20 mmol). The mixture was heated at 70 °C overnight and then extracted with dichloromethane (4 × 100 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and rotary evaporated to give a brown oil (2.27 g, 88%). ^{1}H NMR (CDCl$_3$) δ: 2.20 (s, 3H), 2.95 (t, J = 6 Hz, 2H), 3.85 (t, J = 6 Hz, 2H), 5.16 (s, 2H), 6.32 (d, J = 7.8 Hz, 1H), 7.31 (d, J = 7.8 Hz, 1H), 7.34 (s, 5H).

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9a). To a vigorously stirred suspension of **3a** (2.34 g, 10 mmol) in dichloromethane (100 mL) were added dicyclohexylcarbodiimide (DCCI) (2.3 g, 11 mmol), 2-mercaptothiazoline (1.32 g, 11 mmol), and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) (50 mg). The mixture was stirred for 24 h, the white precipitate *N,N′*-dicyclohexylurea (DCU) filtered from the yellow solution, the filtrate added to the amine **8** (2.58 g, 10 mmol), and the reaction mixture allowed to stir overnight. The dichloromethane layer was washed with 0.1 N sodium hydroxide solution (3 × 50 mL) and water (50 mL) and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. The crude product was further purified by column chromatography on silica gel (eluant: methanol:chloroform, 1:9 v/v) to afford a white solid (2.32 g, 49%): mp 202−203 °C; ^{1}H NMR (CDCl$_3$) δ: 2.05 (s, 3H), 3.54 (s, 2H), 3.72−3.84 (m, 7H), 4.81 (s, 2H), 5.90 (d, J = 7.8 Hz, 1H), 6.06 (s, 1H), 6.70−6.85 (m, 2H), 7.18 (s, 5H), 7.38 (d, J = 9.0 Hz, 1H), 8.10 (d, J = 7.8 Hz, 1H). *m/z*: 475 (M + 1)[+].

Analogous procedures starting with coumarin acids with amino derivatives gave **9b−f**, **9h**, **9k−m**, **9o−v**.

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-2-(6,7-dimethoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9b): (2.67 g, 53%); mp 164−166 °C; ^{1}H NMR (CDCl$_3$) δ: 2.15 (s, 3H), 3.65 (s, 2H), 3.70−3.90 (m, 10H), 4.90 (s, 2H), 5.95 (d, J = 7.8 Hz, 1H), 6.08 (s, 1H), 7.05 (s, 1H), 7.12 (s, 1H), 7.20 (s, 5H), 8.01 (d, J = 7.8 Hz, 1H). *m/z*: 505 (M + 1)[+].

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-2-(7-anilino-2-oxo-2*H*-chromen-4-yl)acetamide (9c): (2.94 g, 55%); mp 146−148 °C; ^{1}H NMR (CDCl$_3$) δ: 2.15 (s, 3H), 2.90−3.25 (m, 4H), 4.55 (s, 2H), 5.24 (s, 2H), 6.16−8.58 (m, 16H). *m/z*: 536 (M + 1)[+].

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamide (9d): (2.63 g, 54%); mp 210−211 °C; ^{1}H NMR (CDCl$_3$) δ: 2.12 (s, 3H), 3.00 (s, 6H), 3.35−3.80 (m, 6H), 4.98 (s, 2H), 5.99 (s, 1H), 6.30 (d, J = 7.8 Hz, 1H), 6.40−6.70 (m, 2H), 7.20 (s, 5H), 7.30 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 7.2 Hz, 1H). *m/z*: 488 (M + 1)[+].

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-7-methoxy-2-oxo-2*H*-chromen-3-carboxamide (9e): (2.61 g, 58%); mp 182−183 °C; ^{1}H NMR (CDCl$_3$) δ: 2.18 (s, 3H), 3.61 (q, J = 6.5 Hz, 2H), 3.93 (s, 3H), 4.01 (t, J = 6.8 Hz, 2H), 5.21 (s, 2H), 6.41 (d, J = 7.5 Hz, 1H), 6.88 (d, J = 2.3 Hz, 1H), 6.97 (dd, J = 2.3, 8.7 Hz, 1H), 7.23 (d, J = 7.5 Hz, 1H), 7.26−7.43 (m, 5H), 7.60 (d, J = 8.7 Hz, 1H), 8.80 (s, 1H), 8.95 (t, J = 5.9 Hz, 1H). *m/z*: 461 (M + 1)[+].

N-[2-(3-Benzoxy-2-methyl-4-oxopyridin-1(4*H*)-yl)ethyl]-7-diethylamino-2-oxo-2*H*-chromene-3-carboxamide (9f): (2.45 g, 49%); mp 208−209 °C; ^{1}H NMR (CDCl$_3$) δ: 1.25 (t, J = 7.1 Hz, 6H), 2.18 (s, 3H), 3.46 (q, J = 7.1 Hz, 4H), 3.58 (q, J = 6.6 Hz, 2H), 3.99 (t, J = 6.8 Hz, 2H), 5.22 (s, 2H), 6.40 (d, J = 7.5 Hz, 1H), 6.50 (d, J = 2.3 Hz, 1H), 6.67 (dd, J = 2.3, 9.0 Hz, 1H), 7.22 (d, J = 7.5 Hz, 1H), 7.27−7.45 (m, 6H), 8.65 (s, 1H), 8.99 (t, J = 5.9 Hz, 1H). *m/z*: 502 (M + 1)[+].

7-Diethylamino-N-[(5-methoxy-6-methyl-4-oxo-1,4-dihydropyridin-3-yl)methyl]-N-methyl-2-oxo-2*H*-chromene-3-carboxamide (9h): (2.72 g, 64%); mp 201−202 °C; ^{1}H NMR (CDCl$_3$) δ: 1.12 (t, J = 6.6 Hz, 6H), 2.20 (s, 3H), 3.00 (s, 3H),

3.31 (q, J = 6.6 Hz, 4H), 3.70 (s, 3H), 4.48 (s, 2H), 6.25−6.55 (m, 2H), 7.14 (d, J = 7.2 Hz, 1H), 7.63 (s, 2H). *m/z*: 426 (M + 1)[+].

N-[(5-Benzoxy-4-oxo-4*H*-pyran-2-yl)methyl]-2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9k): (2.23 g, 50%); mp 206−207 °C; ^{1}H NMR (CDCl$_3$) δ: 3.77 (s, 2H), 3.80 (s, 3H), 4.09 (d, J = 5.8 Hz, 2H), 4.82 (s, 2H), 6.12 (s, 1H), 6.15 (s, 1H), 6.70−6.90 (m, 2H), 7.25 (s, 5H), 7.55 (d, J = 8.8 Hz, 1H), 8.00 (s, 1H), 8.68 (br s, 1H). *m/z*: 448 (M + 1)[+].

N-[(5-Benzoxy-4-oxo-4*H*-pyran-2-yl)methyl]-2-(6,7-dimethoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9l): (2.62 g, 55%); mp 230−231 °C; ^{1}H NMR (CDCl$_3$) δ: 3.78 (s, 3H), 3.83 (s, 2H), 3.87 (s, 3H), 4.19 (d, J = 5.8 Hz, 2H), 4.93 (s, 2H), 6.25 (s, 1H), 6.29 (s, 1H), 7.09 (s, 1H), 7.17 (s, 1H), 7.34−7.42 (m, 5H), 8.13 (s, 1H), 8.82 (d, J = 5.8 Hz, 2H). *m/z*: 478 (M + 1)[+].

7-Methoxy-N-[(5-benzoxy-4-oxo-4*H*-pyran-2-yl)methyl]-2-oxo-2*H*-chromene-3-carboxyamide (9m): (2.08 g, 48%); mp 186−188 °C; ^{1}H NMR (CDCl$_3$) δ: 3.80 (s, 3H), 4.32 (d, J = 6.0 Hz, 2H), 4.90 (s, 2H), 6.22 (s, 1H), 6.60−6.85 (m, 2H), 7.20 (s, 5H), 7.34 (s, 1H), 7.55 (d, J = 8.0 Hz, 1H), 8.64 (s, 1H), 9.00 (br s, 1H). *m/z*: 434 (M + 1)[+].

7-Ddiethylamino-N-[(5-benzoxy-1-methyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-2-oxo-2H-chromene-3-carboxamide (9o): (52%); mp 209−210 °C; ^{1}H NMR (CDCl$_3$) δ: 1.25 (t, J = 7.1 Hz, 6H), 3.47 (q, J = 7.1 Hz, 4H), 3.57 (s, 3H), 4.51 (d, J = 6.0 Hz, 2H), 5.17 (s, 2H), 6.51 (d, J = 2.4 Hz, 1H), 6.52 (s, 1H), 6.66 (dd, J = 2.4, 9.0 Hz, 1H), 6.93 (s, 1H), 7.27−7.45 (m, 6H), 8.67 (s, 1H), 9.19 (t, J = 6.0 Hz, 1H). *m/z*: 488 (M + 1)[+].

N-[(3-Benzoxy-6-methyl-4-oxo-4*H*-pyran-2-yl)methyl]-2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9p): (2.07 g, 45%); mp 183−184 °C; ^{1}H NMR (CDCl$_3$) δ: 2.16 (s, 3H), 3.73 (s, 2H), 3.85 (s, 3H), 4.20 (d, J = 5.8 Hz, 2H), 4.99 (s, 2H), 6.19 (s, 1H), 6.23 (s, 1H), 6.23 (s, 1H), 6.80−6.94 (m, 2H), 7.30−7.70 (m, 6H), 8.59 (t, J = 5.6 Hz, 1H). *m/z*: 462 (M + 1)[+].

N-[(3-Benzoxy-6-methyl-4-oxo-4*H*-pyran-2-yl)methyl]-2-(6,7-dimethoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9q): (2.41 g, 49%); mp 224−225 °C; ^{1}H NMR (CDCl$_3$) δ: 2.06 (s, 3H), 3.68 (s, 3H), 3.69 (s, 2H), 3.78 (s, 3H), 4.08−4.20 (m, 2H), 4.90 (s, 2H), 6.08 (s, 1H), 6.15 (s, 1H), 6.90−7.30 (m, 7H), 8.65 (brs, 1H). *m/z*: 492 (M + 1)[+].

N-[(3-Benzoxy-6-methyl-4-oxo-4*H*-pyran-2-yl)methyl]-7-methoxy-2-oxo-2*H*-chromene-3-caroxamide (9r): (2.50 g, 56%); mp 185−186 °C; ^{1}H NMR (CDCl$_3$) δ: 2.20 (s, 3H), 3.84 (s, 3H), 4.40 (d, J = 5.8 Hz, 2H), 5.14 (s, 2H), 6.09 (s, 1H), 6.65−7.65 (m, 9H), 8.70 (s, 1H). *m/z*: 448 (M + 1)[+].

N-[(3-Benzoxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)acetamide (9s): (2.51 g, 53%); mp 244−245 °C; ^{1}H NMR (CDCl$_3$) δ: 2.20 (s, 3H), 3.30 (s, 3H), 3.63 (s, 2H), 3.75 (s, 3H), 4.28 (d J = 4.8 Hz, 2H), 4.96 (s, 2H), 6.05 (s, 1H), 6.10 (s, 1H), 6.70−6.90 (m, 2H), 7.18−7.40 (m, 6H), 8.35 (t, J = 4.8 Hz, 1H). *m/z*: 475 (M + 1)[+].

N-[(3-Benzoxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamide (9t): (2.34 g, 48%); mp 278−279 °C; ^{1}H NMR (CDCl$_3$) δ: 1.24 (t, J = 7.1 Hz, 6H), 2.29 (s, 3H), 3.46(q, J = 7.1 Hz, 4H), 3.57 (s, 3H), 4.63 (d J = 5.7 Hz, 2H), 5.36 (s, 2H), 6.38 (s, 2H), 6.43 (d, J = 2.3 Hz, 1H), 6.49 (dd, J = 2.3, 8.6 Hz, 1H), 7.24−7.50 (m, 6H), 8.64 (s, 1H), 8.78 (t, J = 5.7 Hz, 1H). *m/z*: 488 (M + 1)[+].

N-[(3-Benzoxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-7-methoxy-2-oxo-2*H*-chromene-3-carboxamide (9u): (2.34 g, 52%); mp 186−188 °C; ^{1}H NMR (CDCl$_3$) δ: 2.31 (s, 3H), 3.57 (s, 3H), 3.89 (s, 3H), 4.57 (d J = 5.8 Hz, 2H), 5.31 (s, 2H), 6.29 (s, 1H), 6.70−6.92 (m, 2H), 7.10−7.50 (m, 6H), 8.68 (s, 1H). *m/z*: 461 (M + 1)[+].

N-[(3-Benzoxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-7-diethylamino-2-oxo-2*H*-chromene-3-carboxamide (9v): (2.90 g, 58%); mp 243−244 °C; ^{1}H NMR (CDCl$_3$) δ: 1.13 (t, J = 7.0 Hz, 6H), 2.19 (s, 3H), 3.39 (q, J = 7.0 Hz, 4H), 3.44 (s, 3H), 4.48 (d J = 5.8 Hz, 2H), 5.20 (s, 2H),

6.20 (s, 1H), 6.32–6.60 (m, 2H), 7.02–7.38 (m, 6H), 8.45 (s, 1H). *m/z*: 502 (M + 1)⁺.

N-(2-{[2-(Acetylamino)ethyl][2-(3-benzoxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]amino}ethyl)- 7-diethylamino-2-oxo-2H-chromene-3-carboxamide (9g). To a vigorously stirred suspension of **3f** (5.24 g, 20 mmol) in dichloromethane (150 mL) were added dicyclohexylcarbodiimide (DCCI) (4.6 g, 22 mmol), 2-mercaptothiazoline (2.64 g, 22 mmol), and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) (100 mg). The mixture was stirred for 24 h, the white precipitate *N,N′*-dicyclohexylurea (DCU) filtered from the yellow solution, the filtrate added to the amine **11** (1.72 g, 5 mmol), and the reaction mixture stirred overnight. The compound **9g** was purified following the representative procedure described for **9a** to obtain a yellow solid in 55% yield (2.29 g); mp 189–191 °C; ¹H NMR (CDCl₃) δ: 1.13 (t, *J* = 7.0 Hz, 12 H), 2.58 (s, 3H), 3.46 (q, *J* = 7.0 Hz, 8 H), 3.71 (br s, 12H), 5.05 (s, 2H), 6.53 (s, 2H), 6.74 (dd, *J* = 2.2, 9.1 Hz, 2H), 7.35–7.58 (m, 8H), 8.52–8.58 (m, 3H), 8.88 (br s, 2H). *m/z*: 831 (M + 1)⁺.

4-[2-(5-Benzyloxy-4-oxo-4H-pyran-2-yl)vinyl]-7-methoxychroman-2-one (9i). To a solution of 4-(chloromethyl)-7-methoxy-2H-chromen-2-one **2′a** (4.49 g, 20 mmol) in CHCl₃ (30 mL) was added triphenyl phosphine (5.29 g, 21 mmol), and the mixture was refluxed for 2.5 h. Rotary evaporation to remove the solvent, and the resulting precipitate was isolated by filtration, washed with toluene (10 mL), and dried under high vacuum to yield colorless amorphous powder. The mixture of the former powder (4.86 g, 10 mmol) and 2-formyl-5-benzyloxy-4H-pyran-4-one **18** (2.3 g, 10 mmol) in CH₂Cl₂ (10 mL) was added dropwise aqueous solution of sodium hydroxide (50%, 5 mL) over 15 min and stirred for a further 0.5 h at room temperature. Distilled water (30 mL) was added and extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to yield an brown oil. Further purification by column chromatography on silica gel (eluant: ethyl acetate) and followed by recrystallization from methanol furnished a yellow crystalline solid (32%). mp 177–179 °C; ¹H NMR (CDCl₃) δ: 3.75 (s, 3H), 5.00 (s, 2H), 6.20 (s, 1H), 6.32 (s, 1H), 6.63–7.50 (m, 11H). *m/z*: 403 (M + 1)⁺.

An analogous procedure starting with **2′b** gave **4-[2-(5-benzyloxy-4-oxo-4H-pyran-2-yl)vinyl]-6,7-dimethoxychroman-2-one (9j):** (38%); mp 202–203 °C; ¹H NMR (CDCl₃) δ: 3.88 (s, 6H), 5.01 (s, 2H), 6.27 (s, 1H), 6.38 (s, 1H), 6.72 (s, 1H), 6.80–6.86 (m, 2H), 7.20–7.30 (m, 6H), 7.48 (s, 1H). *m/z*: 433 (M + 1)⁺.

5-Benzyloxy-2-({[(7-methoxy-2-oxo-2H-chromen-4-yl)methyl]amino}methyl)-1-methylpyridin-4(1H)-one (9n). To a solution of **2′a** (3.37 g, 15 mmol) and **23** (3.65 g, 15 mmol) in ethanol (100 mL) was added triethylamine (1.6 g, 16 mmol), and the mixture was heat under reflux for 2 days. After evaporation to remove the solvent, the residue was added 100 mL of water and was extracted with dichloromethane (4 × 100 mL). The organic fraction was then dried over anhydrous sodium sulfate, filtered, and rotary evaporated to yield a pale yellow solid (2.92 g, 45%). mp 121–123 °C; ¹H NMR (CDCl₃) δ: 3.54 (s, 3H), 3.65 (s, 2H), 3.78 (s, 3H), 3.80 (s, 2H), 4.98 (s, 2H), 6.20 (s, 1H), 6.32 (s, 1H), 6.58–6.74 (m, 2H), 6.90 (d, *J* = 2.5 Hz, 1H), 7.20 (s, 5H), 7.30 (s, 1H). *m/z*: 433 (M + 1)⁺.

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-2-(7-methoxy-2-oxo-2H-chromen-4-yl)acetamide Hydrochloride (10a): Method i. Solutions of **9a** in *N,N*-dimethylformamide (DMF) were subjected to hydrogenolysis in the presence of 5% Pd/C (w/w) catalyst for 5 h. The catalysts were removed by filtration, and the filtrates were acidified to pH 1 with concentrated hydrochloric acid. After removal of the solvents in vacuo, the residues were purified by recrystallization from methanol to afford a white solid (89%): mp 256–258 °C; ¹H NMR (DMSO-*d₆*) δ: 2.51 (s, 3H), 3.51 (q, *J* = 5.7 Hz, 2H), 3.69 (s, 2H), 3.87 (s, 3H), 4.39 (t, *J* = 5.7 Hz, 2H), 6.23 (s, 1H), 6.96 (dd, *J* = 2.5, 8.7 Hz, 1H), 6.98 (d, *J* = 2.5 Hz, 1H), 7.19 (d, *J* = 6.9 Hz, 1H), 7.56 (d, *J* = 8.7 Hz, 1H), 8.07 (d, *J* = 6.9 Hz, 1H), 8.61 (t, *J* = 5.9 Hz, 1H). *m/z*: 385 (M − Cl)⁺.

Analogous hydrogenation procedures were used to the preparation for **10b, 10d–10f, 10′j, 10k–10m, 10p–10v.**

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-2-(6,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetamide hydrochloride (10b): (88%); mp 261–262 °C; ¹H NMR (DMSO-*d₆*) δ: 2.50 (s, 3H), 3.51 (q, *J* = 5.8 Hz, 2H), 3.71 (s, 2H), 3.80 (s, 3H), 3.87 (s, 3H), 4.38 (t, *J* = 5.8 Hz, 2H), 6.23 (s, 1H), 7.06 (s, 1H), 7.12 (d, *J* = 7.0 Hz, 1H), 7.19 (s, 1H), 8.06 (d, *J* = 7.0 Hz, 1H), 8.74 (t, *J* = 5.8 Hz, 1H). *m/z*: 415 (M − Cl)⁺.

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide hydrochloride (10d): (82%); mp 258–260 °C; ¹H NMR (DMSO-*d₆*) δ: 2.52 (s, 3H), 3.03 (s, 6H), 3.49–3.53 (m, 2H), 3.61 (s, 2H), 4.40 (t, *J* = 5.6 Hz, 2H), 5.98 (s, 1H), 6.55 (d, *J* = 2.5 Hz, 1H), 6.72 (dd, *J* = 2.5, 9.0 Hz, 1H), 7.28 (d, *J* = 6.9 Hz, 1H), 7.40 (d, *J* = 9.0 Hz, 1H), 8.09 (d, *J* = 6.9 Hz, 1H), 8.58 (t, *J* = 5.8 Hz, 1H). *m/z*: 398 (M − Cl)⁺.

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-7-methoxy-2-oxo-2H-chromene-3-carboxamide hydrochloride (10e): (85%); mp 263–264 °C; ¹H NMR (DMSO-*d₆*) δ: 2.58 (s, 3H), 3.75 (m, 2H), 3.90 (s, 3H), 4.51 (t, *J* = 5.7 Hz, 2H), 7.05 (dd, *J* = 2.4, 8.7 Hz, 1H), 7.11 (d, *J* = 2.4 Hz, 1H), 7.22 (d, *J* = 6.9 Hz, 1H), 7.91 (d, *J* = 8.7 Hz, 1H), 8.04 (d, *J* = 6.9 Hz, 1H), 8.78 (s, 1H), 8.87 (t, *J* = 6.1 Hz, 1H). *m/z*: 370 (M − Cl)⁺.

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-7-diethylamino-2-oxo-2H-chromene-3-carboxamide hydrochloride (10f): (75%); mp 249–251 °C; ¹H NMR (DMSO-*d₆*) δ: 1.13 (t, *J* = 7.0 Hz, 6H), 2.58 (s, 3H), 3.47 (q, *J* = 7.0 Hz), 3.73 (q, *J* = 5.7 Hz, 2H), 4.50 (t, *J* = 5.7 Hz, 2H), 6.59 (d, *J* = 2.3 Hz, 1H), 6.79 (dd, *J* = 2.3, 9.0 Hz, 1H), 7.31 (d, *J* = 7.0 Hz, 1H), 7.66 (d, *J* = 9.0 Hz, 1H), 8.07 (d, *J* = 7.0 Hz, 1H), 8.58 (s, 1H), 8.83 (t, *J* = 5.7 Hz, 1H), 8.93 (brs, 1H). *m/z*: 412 (M − Cl)⁺.

4-[2-(5-Hydroxy-4-oxo-4H-pyran-2-yl)ethyl]-6,7-dimethoxychroman-2-one (10′j): (32%); mp 285–287 °C; ¹H NMR (DMSO-*d₆*) δ:2.70–3.00 (m, 4H), 3.87 (s, 3H), 3.89 (s, 3H), 6.05 (s, 1H), 6.58–6.94 (m, 3H), 7.19 (s, 1H). *m/z*: 345 (M + 1)⁺.

N-[(5-Hydroxy-4-oxo-4H-pyran-2-yl)methyl]-2-(7-methoxy-2-oxo-2H-chromen-4-yl)acetamide (10k): (90%); mp 254–256 °C; ¹H NMR (DMSO-*d₆*) δ: 3.80 (s, 2H), 3.86 (s, 3H), 4.17 (d, *J* = 5.8 Hz, 2H), 6.25 (s, 1H), 6.29 (s, 1H), 6.97 (dd, *J* = 2.6, 8.9 Hz, 1H), 7.01 (d, *J* = 2.6 Hz, 1H), 7.67 (d, *J* = 8.9 Hz, 1H), 8.02 (s, 1H), 8.82 (t, *J* = 5.8 Hz, 1H), 9.13 (s, 1H). *m/z*: 358 (M + 1)⁺.

N-[(5-Hydroxy-4-oxo-4H-pyran-2-yl)methyl]-2-(6,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetamide (10l): (88%); mp 263–265 °C; ¹H NMR (DMSO-*d₆*) δ: 3.79 (s, 3H), 3.83 (s, 2H), 3.87 (s, 3H), 4.19 (d, *J* = 5.8 Hz, 2H), 6.25 (s, 1H), 6.29 (s, 1H), 7.09 (s, 1H), 7.18 (s, 1H), 7.99 (s, 1H), 8.84 (t, *J* = 5.8 Hz, 1H), 9.13 (br s, 1H). *m/z*: 388 (M + 1)⁺.

7-Methoxy-N-[(5-hydroxy-4-oxo-4H-pyran-2-yl)methyl]-2-oxo-2H-chromene-3-carboxyamide (10m): (86%); mp 264–266 °C; ¹H NMR (DMSO-*d₆*) δ: 3.91 (s, 3H), 4.42 (d, *J* = 6.0 Hz, 2H), 6.30 (s, 1H), 7.05 (dd, *J* = 2.4, 8.8 Hz, 1H), 7.13 (d, *J* = 2.4 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 8.06 (s, 1H), 8.84 (s, 1H), 9.13 (br s, 1H). *m/z*: 344 (M + 1)⁺.

N-[(3-Hydroxy-6-methyl-4-oxo-4H-pyran-2-yl)methyl]-2-(7-methoxy-2-oxo-2H-chromen-4-yl)acetamide(10p): (82%); mp 262–263 °C; ¹H NMR (DMSO-*d₆*) δ: 2.17 (s, 3H), 3.73 (s, 2H), 3.85 (s, 3H), 4.29 (d, *J* = 5.5 Hz, 2H), 6.21 (s, 1H), 6.25 (s, 1H), 6.94 (dd, *J* = 2.5, 8.8 Hz, 1H), 7.00 (d, *J* = 2.5 Hz, 1H), 7.67 (d, *J* = 8.8 Hz, 1H), 8.72 (t, *J* = 5.6 Hz, 1H), 9.07 (s, 1H). *m/z*: 372 (M + 1)⁺.

N-[(3-Hydroxy-6-methyl-4-oxo-4H-pyran-2-yl)methyl]-2-(6,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetamide (10q): (89%); mp 261–262 °C; ¹H NMR (DMSO-*d₆*) δ: 2.12 (s, 3H), 3.74 (s, 3H), 3.75 (s, 2H), 3.86 (s, 3H), 4.30 (d, *J* = 5.5 Hz, 2H), 6.21 (s, 1H), 6.26 (s, 1H), 7.09 (s, 1H), 7.19 (s, 1H), 8.83 (t, *J* = 5.5 Hz, 1H). *m/z*: 402 (M + 1)⁺.

N-[(3-Hydroxy-6-methyl-4-oxo-4H-pyran-2-yl)methyl]-7-methoxy-2-oxo-2H-chromene-3-caroxamide (10r): (78%); mp 261–263 °C; ¹H NMR (DMSO-*d₆*) δ: 2.24 (s, 3H), 3.90 (s,

3H), 4.54 (d, $J = 5.8$ Hz, 2H), 6.24 (s, 1H), 7.06 (dd, $J = 2.4$, 8.7 Hz, 1H), 7.13 (d, $J = 2.4$ Hz, 1H), 7.92 (d, $J = 8.7$ Hz, 1H), 8.86 (s, 1H), 9.02 (t, $J = 5.8$ Hz, 1H), 9.14 (s, 1H). m/z: 358 (M + 1)$^+$.

N-[(3-Hydroxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-2-(7-methoxy-2-oxo-2H-chromen-4-yl)acetamide hydrochloride (10s): (82%); mp 250−251 °C; ^{1}H NMR (DMSO-d_6) δ: 2.53 (s, 3H), 3.75 (s, 2H), 3.80 (s, 3H), 3.86 (s, 3H), 4.61 (d $J = 4.9$ Hz, 2H), 6.25 (s, 1H), 6.96 (dd, $J = 2.2$, 8.8 Hz, 1H), 7.01 (s, 1H), 7.02 (d, $J = 2.2$ Hz, 1H), 7.64 (d, $J = 8.8$ Hz, 1H), 8.91 (t, $J = 4.9$ Hz, 1H). m/z: 385 (M − Cl)$^+$.

N-[(3-Hydroxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide hydrochloride (10t): (81%); mp 232−234 °C; ^{1}H NMR (DMSO-d_6) δ: 2.56 (s, 3H), 3.02 (s, 6H), 3.69 (s, 2H), 3.87 (s, 3H), 4.62 (d, $J = 5.1$ Hz, 2H), 6.01 (s, 1H), 6.55 (d, $J = 2.5$ Hz, 1H), 6.71 (dd, $J = 2.5$, 9.0 Hz, 1H), 7.29 (s, 1H), 7.51 (d, $J = 9.0$ Hz, 1H), 9.12 (t, $J = 5.1$ Hz, 1H). m/z: 398 (M − Cl)$^+$.

N-[(3-Hydroxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-7-methoxy-2-oxo-2H-chromene-3-carboxamide hydrochloride (10u): (81%); mp 280−281 °C; ^{1}H NMR (DMSO-d_6) δ: 2.56 (s, 3H), 3.90 (s, 3H), 4.00 (s, 3H), 4.88 (d $J = 5.6$ Hz, 2H), 7.06 (dd, $J = 2.4$, 8.7 Hz, 1H), 7.14 (d, $J = 2.4$ Hz, 1H), 7.21 (s, 1H), 7.91 (d, $J = 8.7$ Hz, 1H), 8.85 (s, 1H), 9.17 (t, $J = 5.6$ Hz, 1H). m/z: 371 (M − Cl)$^+$.

N-[(3-Hydroxy-1,6-dimethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl]-7-diethylamino-2-oxo-2H-chromene-3-carboxamide hydrochloride (10v): (85%); mp 276−277 °C; ^{1}H NMR (DMSO-d_6) δ: 1.13 (t, $J = 7.0$ Hz, 6H), 2.56 (s, 3H), 3.46 (q, $J = 7.0$ Hz, 4H), 4.00 (s, 3H), 4.85 (d $J = 5.8$ Hz, 2H), 6.62 (d, $J = 2.2$ Hz, 1H), 6.81 (dd, $J = 2.2$, 9.1 Hz, 1H), 7.20 (s, 1H), 7.68 (d, $J = 9.1$ Hz, 1H), 8.67 (s, 1H), 9.20 (t, $J = 5.8$ Hz, 1H). m/z: 412 (M − Cl)$^+$.

N-[2-(3-Hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]-2-(7-anilino-2-oxo-2H-chromen-4-yl)acetamide Hydrochloride (10c): Method ii. 9c (1.34 g, 2.5 mmol) was dissolved into CH$_2$Cl$_2$ (50 mL) and flushed with nitrogen. After the flask was cooled to 0 °C, boron trichloride (1M in CH$_2$Cl$_2$, 10 mL) was slowly added, and the reaction mixture was allowed to stir at room temperature for 5 h. The excess BCl$_3$ was eliminated at the end of the reaction by the addition of methanol (10 mL) and left to stir for another 0.5 h. After removal of the solvents under reduced pressure, the residues were purified by recrystallization from methanol/acetone to afford yellow solid (67%); mp 241−242 °C; ^{1}H NMR (CDCl$_3$) δ: 2.51 (s, 3H), 2.95 (m, 2H), 4.13−4.44 (m, 4H), 5.95 (s, 1H), 6.82−8.54 (m, 12H). m/z: 446 (M − 2HCl + 1)$^+$.

Analogous hydrogenation procedures were used to the preparation for **10g−j**, **10n−o**.

N-(2-{[2-(Acetylamino)ethyl][2-(3-hydroxy-2-methyl-4-oxopyridin-1(4H)-yl)ethyl]amino}ethyl)-7-diethylamino-2-oxo-2H-chromene-3-carboxamide hydrochloride (10g): (74%); mp 186−188 °C; ^{1}H NMR (DMSO-d_6) δ: 1.13 (t, $J = 7.0$ Hz, 12H), 2.56 (s, 3H), 3.47 (q, $J = 7.0$ Hz, 8H), 3.50 (br s, 12H), 6.54 (s, 2H), 6.74 (dd, $J = 2.2$, 9.1 Hz, 2H), 7.30 (d, $J = 6.9$ Hz, 1H), 7.57 (d, $J = 9.1$ Hz, 2H), 8.31 (d, $J = 6.9$ Hz, 1H), 8.53 (s, 2H), 8.91 (br s, 2H). m/z: 741 (M − 2HCl + 1)$^+$.

7-Diethylamino-N-[(5-hydroxy-6-methyl-4-oxo-1,4-dihydropyridin-3-yl)methyl]-N-methyl-2-oxo-2H-chromene-3-carboxamide hydrochloride (10h): (75%); mp 209−210 °C; ^{1}H NMR (DMSO-d_6) δ: 1.14 (t, $J = 6.9$ Hz, 6H), 2.54 (s, 3H), 3.01 (s, 3H), 3.47 (q, $J = 6.9$ Hz, 4H), 4.66 (s, 2H), 6.61 (d, $J = 2.0$ Hz, 1H), 6.78 (dd, $J = 2.0$, 8.9 Hz, 1H), 7.54 (d, $J = 8.9$ Hz, 1H), 8.09 (s, 2H); m/z: 412 (M − Cl)$^+$.

4-[2-(5-Hydroxy-4-oxo-4H-pyran-2-yl)vinyl]-7-methoxy-chroman-2-one (10i): (67%); mp 268−269 °C; ^{1}H NMR (DMSO-d_6) δ: 3.70 (s, 3H), 6.27−7.84 (m, 8H). m/z: 313 (M + 1)$^+$.

4-[2-(5-Hydroxy-4-oxo-4H-pyran-2-yl)vinyl]-6,7-dimethoxychroman-2-one (10j): (70%); mp 278−280 °C; ^{1}H NMR (DMSO-d_6) δ: 3.88 (s, 3H), 3.89 (s, 3H), 6.51−8.13 (m, 7H). m/z: 343 (M + 1)$^+$.

5-Hydroxy-2-({[(7-methoxy-2-oxo-2H-chromen-4-yl)-methyl]amino}methyl)-1-methylpyridin-4(1H)-one hydrochloride (10n): (60%); mp 234−236 °C; ^{1}H NMR (DMSO-d_6) δ: 3.60 (br s, 1H), 3.88 (s, 3H), 4.20 (s, 3H), 4.55 (s, 2H), 4.61 (s, 2H), 6.66 (s, 1H), 7.03 (dd, $J = 2.5$, 8.8 Hz, 1H), 7.07 (d, $J = 2.5$ Hz, 1H), 7.72 (s, 1H), 7.80 (d, $J = 8.8$ Hz, 1H), 8.39 (s, 1H). m/z: 343 (M − 2HCl + 1)$^+$.

7-Diethylamino-N-[(5-hydroxy-1-methyl-4-oxo-1,4-di-hydropyridin-2-yl)methyl]-2-oxo-2H-chromene-3-carbox-amide hydrochloride (10o): (72%); mp 246−248 °C; ^{1}H NMR (DMSO-d_6) δ: 1.15 (t, $J = 7.0$ Hz, 6H), 3.50 (q, $J = 7.0$ Hz, 4H), 4.07 (s, 3H), 4.71 (d, $J = 5.9$ Hz, 2H), 6.65 (d, $J = 2.0$ Hz, 1H), 6.83 (dd, $J = 2.0$, 9.0 Hz, 1H), 7.22 (s, 1H), 7.71 (d, $J = 9.0$ Hz, 1H), 8.28 (s, 1H), 8.69 (s, 1H), 9.27 (t, $J = 5.9$ Hz, 1H); m/z: 398 (M − Cl)$^+$.

An analogous reaction of 3-benzyloxy-2-methylpyran-4-one with tris(2-aminoethyl)amine as described for **8** gave brown oil **1-{2-[bis(2-Aminoethyl)amino]ethyl}-3-benzoxy-2-methylpyridin-4(1H)-one (11):** (2.92 g, 85%); ^{1}H NMR (CDCl$_3$) δ: 2.05 (s, 3H), 2.35 (m, 10H),3.20 (br s, 4H), 3.70 (t, $J = 7.2$ Hz, 2H), 4.84 (s, 2H), 6.02 (d, $J = 7.2$ Hz, 1H), 7.18 (s, 5H), 7.44 (d, $J = 7.2$ Hz, 1H).

2-Methyl-3-methoxypyran-4(1H)-one (12). To a solution of methyl maltol (6) (12.6 g, 0.1 mol) in 62 mL of 10% potassium hydroxide was added redistilled dimethyl sulfate (13.9 g, 0.11 mol) in portions over a period of 30 min. The temperature was kept below 25 °C by occasional cooling in an ice-bath. Stirring was continued for an additional 1 h, and the mixture was extracted with dichloromethane (3 × 100 mL). The combined extracts were washed with 5% aqueous potassium hydroxide (2 × 100 mL) followed by water (3 × 100 mL). The organic fraction was then dried over anhydrous sodium sulfate, filtered, and rotary evaporated to yield an orange oil. (10.4 g, 74%); ^{1}H NMR (CDCl$_3$) δ: 2.10 (s, 3H), 3.80 (s, 3H), 6.22 (d, $J = 6.0$ Hz, 1H), 7.60 (d, $J = 6.0$ Hz, 1H).

An analogous reaction of 3-methoxy-2-methylpyran-4-one with ammonia as described for **8** gave **3-methoxy-2-meth-ylpyridin-4(1H)-one (13):** (1.32 g, 95%); mp 153−154 °C (lit. value[36] 155−156.5 °C); ^{1}H NMR (CDCl$_3$) δ: 2.35 (s, 3H), 3.76 (s, 3H), 6.30 (d, $J = 7.8$ Hz, 1H), 7.34 (d, $J = 7.8$ Hz, 1H).

5-{[Benzyl(methyl)amino]methyl}-3-methoxy-2-meth-ylpyridin-4(1H)-one (14). Benzylmethylamine (4.84 g, 40 mmol) and 40% formaldehyde (1.5 g, 20 mmol) were added to 100 mL of ethanol, followed by 3-methoxy-2-methyl-4(1H)-pyridinone (1.4 g, 10 mmol). The mixture was refluxed for 24 h and then rotary evaporated to an oil which crystallized on standing. The crude product was recrystallized from acetone/ethanol to afford colorless solid (2.67 g, 98%). mp 138−139 °C; ^{1}H NMR (DMSO-d_6) δ: 2.20 (s, 3H), 2.38 (s, 3H), 3.55 (s, 2H), 3.63 (s, 2H), 3.78 (s, 3H), 7.21 (s, 5H), 7.70 (s, 1H).

3-Methoxy-2-methyl-5-[(methylamino)methyl]pyridin-4(1H)-one (15): Solutions of **14** in EtOH were subjected to hydrogenolysis in the presence of 5% Pd/C (w/w) catalyst for 4 h. The catalysts were removed by filtration. After removal of the solvents in vacuo, the residues were dissolved in dichloromethane and cooled for 2 days to afford a white solid (99%); mp 99−108 °C; ^{1}H NMR (DMSO-d_6) δ: 2.12 (s, 3H), 2.37 (s, 3H) 3.61 (s, 2H), 3.77 (s, 3H), 7.20 (br s, 1H), 7.46 (s, 1H).

5-(Benzyloxy)-2-(hydroxymethyl)-4H-pyran-4-one (17). To a solution of kojic acid **16** (71 g, 0.5 mol) in methanol (500 mL) was added sodium hydroxide (22 g, 0.55 mol) dissolved in water (50 mL), and the mixture was heated to reflux. Benzyl chloride (70 g, 0.55 mol) was added dropwise over 30 min, and the resulting mixture was refluxed overnight. After removal of solvent by rotary evaporation, the brown solid was washed with water (200 mL) followed by methanol (100 mL). Recrystallization from methanol gave the pure product as colorless needles (90.5 g, 78%); mp 132−133 °C (lit. value[37] 132 °C); ^{1}H NMR (DMSO-d_6) δ: 4.17 (s, 2H), 4.80 (s, 2H), 5.52 (br s, 1H), 6.14 (s, 1H), 7.16 (s, 5H), 7.89 (s, 1H).

2-Formyl-5-benzyloxy-4H-pyran-4-one (18). To a solution of **17** (5.0 g, 21.5 mmol) in chloroform (100 mL) were added dimethyl sulfoxide (27 mL) and triethylamine (18.5 mL), and

the reaction mixture was cooled with an ice-bath to an internal temperature of 3−5 °C. Then sulfur trioxide pyridine complex (17.1 g, 107 mmol) was added, and the mixture was allowed to thaw to room temperature. After stirring for 12 h at room temperature, the reaction mixture was washed with water (2 × 50 mL), and the organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to yield a yellow solid. Further purification by column chromatography (eluent: EtOAc) furnished a pale yellow solid (3.9 g, 79%). mp 130−132 °C (lit. value[38] 133−135 °C); ^{1}H NMR (CDCl$_3$) δ: 5.00 (s, 2H), 6.85 (s, 1H), 7.25 (s, 5H), 7.57 (s, 1H), 9.53 (s, 1H).

2-Phthalimidomethyl-5-benzyloxy-pyran-4-one (19). To a solution of triphenylphosphine (6.3 g, 24 mmol) and phthalimide (3.5 g, 24 mmol) in THF (100 mL) was added 2-hydroxymethyl-5-benzyloxypyran-4-one 17 (4.64 g, 20 mmol), and the mixture was cooled to 0 °C in an ice bath. Diethyl azodicarboxylate (4.2 g, 24 mmol) was added dropwise with stirring over 30 min, after which the reaction mixture was allowed to warm slowly to room temperature and to stand overnight. The resulting precipitate was isolated by filtration, washed with THF (5 mL), and dried under high vacuum to yield a white amorphous powder (4.8 g, 66%); mp 192−193 °C; ^{1}H NMR (DMSO-d_6) δ: 4.26 (s, 2H), 4.55 (s, 2H), 6.14 (s, 1H), 6.96 (s, 5H), 7.33 (s, 4H), 7.84 (s, 1H).

2-Aminomethyl-5-benzyloxy-pyran-4-one (20). To a solution of **19** (5.4 g, 15 mmol) in ethanol (96% 50 mL) was added hydrazine monohydrate (0.8 g, 16 mmol). After being refluxed for 3 h, the reaction mixture was chilled to 0 °C and acidified to pH 1 with concentrated in vacuo, and the residue was redissolved in distilled water (50 mL), adjusted to pH 12 with 10 N sodium hydroxide, and extracted with dichloromethane (3 × 100 mL). The combined organic extracts were dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to yield brown oil. Purification by column chromatography on silica gel furnished orange oil (2.32 g, 67%); mp 81−83 °C (lit. value[39] 84.5−86.5 °C); ^{1}H NMR (CDCl$_3$) δ: 2.98 (br s 2H), 3.50 (s 2H), 4.90 (s, 2H), 6.20 (s, 1H), 7.18 (s, 5H), 7.33 (s, 1H).

An analogous reaction of protected kojic acid with methylamine as described for **8** gave **5-(benzyloxy)-2-(hydroxymethyl)-1-methylpyridin-4(1*H*)-one (21):** (1.76 g, 72%); mp 215.5−217.5 °C (lit. value[40] 213-217 °C); ^{1}H NMR (CDCl$_3$) δ: 3.39 (s, 3H), 4.09 (s, 2H), 4.71 (s, 2H), 5.93 (s, 1H), 7.08 (s, 5H), 7.24 (s, 1H).

1-Methyl-2-chloromethyl-5-benzyloxypyridin-4(1*H*)-one hydrochloride (22). 1-Methyl-2-hydroxymethyl-5-benzyloxypyridin-4(1*H*)-one **21** (8.54 g, 35 mmol) was dissolved in distilled thionyl chloride (20 mL) and stirred for 1 h, after which time a pale yellow crystalline mass formed. The product was collected by filtration and washed with petroleum ether to afford colorless amorphous powder (9.4 g, 90%). mp 165.5−166 °C; ^{1}H NMR (DMSO-d_6) δ: 4.10 (s, 3H), 4.82 (s, 2H), 4.95 (s, 2H), 7.12 (s, 5H), 7.40 (s, 1H), 8.66 (s, 1H).

1-Methyl-2-aminomethyl-5-benzyloxypyridin-4(1*H*)-one (23). To a solution of **22** (6.0 g, 20 mmol) in distilled water (100 mL) was added ammonia (40%, 10 mL) and was stirred overnight at room temperature. After removal of water by rotary evaporation, the residue was extracted with hot 2-propanol (100 mL) and was filtered. The filtrate was concentrated in vacuo to yield pale yellow solid (3.94 g, 81%). mp 184−187 °C; ^{1}H NMR (DMSO-d_6) δ: 2.50 (br s, 2H), 3.42 (s, 2H), 3.48 (s, 3H), 4.78 (s, 2H), 6.04 (s, 1H), 7.14 (s, 5H), 7.25 (s, 1H).

An analogous procedure starting with **16** as described for **22** gave **2-chloromethyl-5-hydroxypyran-4(1*H*)-one (24):** (80%); mp 164−164.5 °C (lit. value[41] 166−168 °C); ^{1}H NMR (DMSO-d_6) δ: 4.65 (s, 2H), 6.62 (s, 1H), 8.23 (s, 1H), 8.80 (br s, 1H).

2-Methyl-5-hydroxypyran-4(1*H*)-one (25). Chlorokojic acid **24** (30 g, 0.187 mol) was added to 100 mL of distilled water and heated to 50 °C with stirring. Zinc dust (24.4 g, 0.375 mol) was added followed by the dropwise addition of concentrated hydrochloric acid (56.1 mL) over 1 h with vigorous stirring maintaining the temperature between 70 and 80 °C. The reaction mixture was stirred for a further 3 h at 70 °C. The

excess zinc was removed by hot filtration, and the filtrate was extracted with dichloromethane (5 × 200 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to yield the crude product. Recrystallization from 2-propanol afforded colorless plates (17.4 g, 74%). mp 149−150 °C (lit. value[41] 152−153 °C); ^{1}H NMR (DMSO-d_6) δ: 2.25 (s, 3H), 6.14 (s, 1H), 6.70 (br s, 1H), 7.63 (s, 1H).

2-Hydroxymethyl-3-hydroxy-6-methyl-pyran-4(1*H*)-one (26). Allomaltol **25** (12.6 g, 100 mmol) was added to an aqueous solution of sodium hydroxide (4.4 g, 110 mmol) in distilled water (100 mL) and stirred at room temperature for 5 min. 35% Formaldehyde solution (9 mL) was added dropwise over 10 min and the solution allowed stirred for 12 h. Acidification to pH 1 using concentrated hydrochloric acid and cooling to 3−5 °C for 12 h gave a crystalline deposit (14.4 g, 92%); mp 157 °C (lit. value[41] 159−161 °C); ^{1}H NMR (DMSO-d_6) δ: 2.23 (s, 3H), 4.38 (s, 2H), 4.90 (br s, 1H), 6.20 (s, 1H), 8.80 (br s, 1H).

An analogous procedure starting with **26** as described for **7** gave **2-(hydroxymethyl)-3-benzyloxy-6-methyl-4*H*-pyran-4-one (27a):** (80%); mp 113−114 °C (lit. value[42] 115−116 °C). ^{1}H NMR (CDCl$_3$) δ: 2.20 (s, 3H), 2.63 (br s, 1H), 4.28 (s, 2H), 5.15 (s, 2H), 6.15 (s, 1H), 7.36 (s, 5H).

1,6-Dimethyl-2-hydroxymethyl-3-benzyloxypyridin-4(1*H*)-one (27b). To a solution of **27a** (2.46 g, 10 mmol) in dichloromethane (50 mL) was added 3,4-dihydro-2*H*-pyran (1.7 g, 20 mmol) followed by *p*-toluenesulfonic acid monhydrate (30 mg). After being stirred at room temperature for 3 h, the reaction mixture was washed with 5% aqueous sodium carbonate (20 mL) followed by water (2 × 20 mL). The organic fraction was then rotary evaporated to yield light yellow oil, which was then dissolved in water (50 mL)/40% aqueous methylamine (10 mL). The reaction mixture was heated at 70 °C overnight. After the solution was concentrated ∼2-fold by rotary evaporation, the residue was redissolved in 20 mL of water and was adjusted pH 1 with concentrated hydrochloric acid and refluxed for 4 h, followed by extraction into dichloromethane (4 × 50 mL), and the combined organic layers were then dried over anhydrous sodium sulfate, filtered, and rotary evaporate to give a light brown solid. Recrystallization from methanol/diethyl ether afforded the pure product as a white crystalline solid (2.02 g, 79%); mp 182.5−184 °C (lit. value[42] 181−183 °C); ^{1}H NMR (CDCl$_3$) δ: 2.13 (s, 3H), 3.42 (s, 3H), 4.42 (s, 2H), 4.83 (s, 2H), 5.95 (s, 1H), 7.18 (s, 5H).

An analogous procedure starting with **27a** as described for **19** gave **6-methyl-2-phthalimidomethyl-3-benzyloxypyran-4-one (28a):** (53%); mp 223−225 °C; ^{1}H NMR (CDCl$_3$) δ: 2.10 (s, 3H), 4.64 (s, 2H), 5.16 (s, 2H), 6.03 (s, 1H), 7.10−7.70 (m, 9H).

An analogous procedure starting with **27b** as described for **19** gave **1,6-dimethyl-2-phthalimidomethyl-3-benzyloxypyridin-4(1*H*)-one (28b):** (80%); mp 250.5−252.5 °C (lit. value[42] 250 °C); ^{1}H NMR (DMSO-d_6) δ: 2.20 (s, 3H), 3.43 (s, 3H), 4.80 (s, 2H), 5.08 (s, 2H), 6.08 (s, 1H), 7.23 (s, 5H), 7.71 (s, 4H).

An analogous procedure starting with **28a** as described for **20** gave **2-aminomethyl-6-methyl-3-benzyloxy-pyran-4-one (29a)** as a brown oil: (65%); ^{1}H NMR (CDCl$_3$) δ: 2.17 (s, 3H), 3.41 (s, 2H), 5.05 (s, 2H), 6.02 (s, 1H), 7.20 (s, 5H).

An analogous procedure starting with **28b** as described for **20** gave **1,6-dimethyl-2-aminomethyl-3-benzyloxypyridin-4(1*H*)-one (29b):** (88%); mp 140−142 °C (lit. value[42] 143−144 °C); C. ^{1}H NMR (CDCl$_3$) δ: 2.20 (s, 3H), 3.54 (s, 3H), 3.78 (s, 2H), 5.26 (s, 2H), 6.24 (s, 1H), 7.30 (s, 5H).

Fluorescence Methods. Optical Characterization. The UV/vis spectra of the two probes were recorded using a Perkin-Elmer spectrophotometer (type UV/VIS Lambda 2S), and a Perkin-Elmer spectrofluorometer (type LS 50B) was used to record fluorescence emission spectra operating at a scan rate of 120 nm/min. Spectra were not corrected for light intensity or detector sensitivity. Data were recorded on-line and analyzed by Excel software on a PC computer. Fluorescence

quenching measurements were performed in MOPS buffer (pH 7.4) at 22 °C.

Recording of Fluorescence Quantum Yield. The fluorescence quantum yield (Φ_F), defined as the ratio of photons absorbed to photons emitted by fluorescence, gives the probability of the excited-state being deactivated by fluorescence rather than by another, nonradiative mechanism. The most reliable method for recording Φ_F is the comparative method of Williams et al. 1983,[43] which involves the use of well characterized standard samples with known Φ_F values. For these measurements, quinine and harmane were adopted as standards because they absorb at the excitation wavelength appropriate to the samples and emit in similar spectral regions. To minimize reabsorption effects,[44] absorbances in the 10 mm fluorescence cuvette should never exceed 0.1 at and above the excitation wavelength. Above this level, nonlinear effects may be observed due to inner filter effects. Spectroscopic grade solvents were used and checked for background fluorescence. The fluorescence of each sample was recorded in a range of five concentrations (Absorbance values in the range 0.02−0.1), and plots of integrated fluorescence intensity (I_F) vs absorbance (A) were recorded.

The gradient of each plot (I_F vs A) is proportional to the quantum yield of the sample. Absolute values of quantum yield were calculated using standard samples which have a known fluorescence quantum yield, given by the following equation:

$$\Phi_x = \Phi_{ST}\left(\frac{\text{Grad}_x}{\text{Grad}_{ST}}\right)\left(\frac{\eta^2_x}{\eta^2_{ST}}\right)$$

where the subscripts ST and X denote standard and test respectively, Φ is the fluorescence quantum yield, Grad the gradient from the plot of integrated fluorescence intensity vs absorbance, and η the refractive index of the solvent.

The two standard compounds were cross-calibrated using this equation. This was achieved by calculating the quantum yield of each standard sample relative to the other. Once the standard samples are cross-calibrated, the quantum yield values for the test samples were then calculated, using the same equation. For each test sample, two Φ_F values were obtained relevant to each of the two standards. The average of these two values represents the quantum yield of the test sample and is the value reported in this study.

Membrane Permeability Studies. (a) Preparation of Erythrocyte Ghosts. Freshly drawn human blood (30 mL) was collected into heparinized tubes from volunteers with consent and stored at 4 °C for a maximum of 3 days. Hemoglobin-free erythrocyte ghosts were prepared by gel filtration chromatography.[45] Blood (5−6 mL) was centrifuged (1000g) for 10 min, the plasma and white cells were removed by aspiration, and the red cells were resuspended at 0 °C in isotonic HEPES buffer (20 mM HEPES, 146 mM NaCl, pH 7.4). This procedure was repeated four times. After the final wash, the supernatant was aspirated, and the packed red cells ($\approx$3 mL) were lysed with hypotonic buffer (15 mM PIPES, 0.1 mM EDTA pH 6, and approximately 50 mOsm) at a 10% hematocrit. The suspension was gently shaken and cooled in an ice bath for 5 min before loading onto a Sepharose 2B size-exclusion column (5 × 28 cm) preequilibrated with the hypotonic PIPES buffer and maintained at 0 °C by a cooling jacket with circulating antifreeze. The column was eluted with HEPES buffer at a constant flow rate of 30 mL·h^{-1}, and fractions were collected in tubes in an ice bath to prevent resealing. The white ghosts were eluted in the void volume (70 mL) well removed from the main hemoglobin band eluting at about 130 mL. The lysed cells were collected by centrifugation (11 000g, 10min, 0 °C), the supernatant aspirated and the pellet resuspended in isotonic HEPES buffer at 0 °C to prevent resealing. This washing procedure was repeated four times, to replace the hypotonic buffer, and all operations were performed at 0 °C to prevent the ghost cells from resealing.

(b) Resealing Ghosts. After the final wash the supernatant was aspirated and the packed ghost cells were suspended in 5 mL of resealing buffer, consisting of 25 mM MOPS (pH 7.4), 1 mM ascorbic acid ,and 100 μM Fe^{2+}, at 0 °C. The resuspended membranes were placed on ice for 10 min and then incubated at 37 °C for 30 min to reseal the lysed ghost cells. After resealing, the ghost cells were washed three times in MOPS buffer (25 mM MOPS, 1 mM ascorbic acid) by successive centrifugation (10 000g, 5 min) and resuspension in fresh buffer.

(c) Permeability Measurements. Resealed ghost cells were resuspended in 1 mL of MOPS buffer (25 mM MOPS, 1 mM ascorbic acid) containing either 15 μM **10a** or 6 μM **10v**, without and with 0.1mM DTPA. Fluorescence intensity was recorded during 30 min incubation at 20 °C using a Perkin-Elmer luminescence spectrometer. The results were digitized and subjected to analysis using MicroCal v6 software.

Note Added after ASAP Posting. A revised Figure 3 and caption, with **10h** and **10v** transposed, were added to the version of the manuscript posted October 30, 2004. The new version was posted November 8, 2004.

Supporting Information Available: Table of elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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