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CHEMICAL FEASIBILITY STUDIES OF A POTENTIAL COUMARIN-BASED PRODRUG SYSTEM

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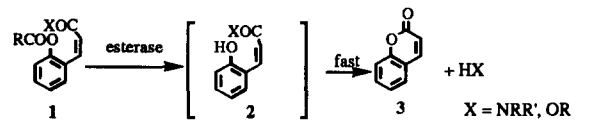
Abstract. By using model amines, several amides of coumarinic acid with the phenolic hydroxyl group protected as an ester were prepared. These model amides underwent a facile ($t_{1/2}$ 1.5–31 min) lactonization to release the original amine compounds upon esterase catalyzed hydrolysis of the phenolic ester. Such a system can be used for the preparation of esterase-sensitive prodrugs of amine-containing compounds or peptides.

Introduction

Prodrug strategies are important for the improvement of the undesirable physico-chemical and biological properties of many drugs.[2–6] Recently, prodrug strategies have found new applications in antibody-directed targeted drug delivery.[7–11] One critical requirement of any prodrug strategy is that it has to be bioreversible, namely, the original drug can be released at the desired time and site in a biological system. This bioreversible masking of functional groups is relatively easy to achieve if the functional groups in need of modification are either hydroxyl or carboxyl groups. These functional groups can be converted to esters, which can be cleaved in a biological system by esterases. However, applying similar strategies to the preparation of prodrugs of amine-containing drugs is somewhat more difficult. This is because of the relative chemical and enzymatic stability of an amide bond in the biological system, which makes the regeneration of the original drug difficult.[2,3] There have been several attempts to develop esterase-sensitive prodrugs of amine-containing compounds by using systems poised to undergo intramolecular cyclization with concomitant release of the amine compounds upon esterase-catalyzed regeneration of a free hydroxyl group.[2,12–17] For example, a masked γ-hydroxy carboxylic acid can be used to make an amide of an amino functionality. Upon unmasking of the hydroxyl group, the system undergoes a spontaneous lactonization to release the amine-containing compound. However, most of these approaches have resulted in amine release rates that are still considered relatively slow at physiological pH. Borchardt and co-workers have developed an esterase-sensitive prodrug system for amine-containing drugs, which can rapidly release the amine drug upon unmasking of a phenolic hydroxyl group.[12,13,17,18] The design takes advantage of a "trimethyl lock" facilitated facile lactonization system.[19–22]

We report the chemical feasibility studies of a potential coumarin-based prodrug system for amine-containing drugs. The design takes advantage of the facile cyclization reaction of coumarinic acid (**2**, X = OH) and its derivatives.[23–26] Because of the presence of a phenolic hydroxyl group and the *cis*-geometry of the double bond, coumarinic acid and derivatives can undergo a facile lactonization at rates comparable to that of the "trimethyl lock" system.[23,24] For example, the effective molarity of the coumarinic acid system with regard to the ester formation (lactonization) reaction is about 3.7×10^{11} compared with the effective molarity of 7×10^{11} of the "trimethyl lock" system.[23,24] With the introduction of other substituents, the effective molarity of the coumarinic acid system can be even higher than that of the "trimethyl lock" system. The phenol hydroxyl group of coumarinic acid can be converted to an ester to serve as an esterase-sensitive biological trigger for the release of the amine-containing drugs (Scheme 1). The basic design principle of this coumarin-based prodrug system is

similar to that of the "trimethyl lock" facilitated facile lactonization system. However, several reasons justify the development of this coumarin-based prodrug system. First, the final product of the pro-moiety after the drug is released is coumarin (**3**), of which the toxicity has been extensively studied and was found to be relatively non-toxic.[27–31] The availability of the toxicity data of coumarin eliminates one major uncertainty with the clinical development of any prodrug moiety. Secondly, the synthesis of coumarin-based prodrugs starts from a readily available, inexpensive material, coumarin. Third, the coumarin-based prodrugs, because of their structural differences compared with the "trimethyl lock"-base prodrug system, are expected to have somewhat different release kinetics and enzyme (esterase) specificity, which would complement the "trimethyl lock"-based prodrug system in terms of potential applications.



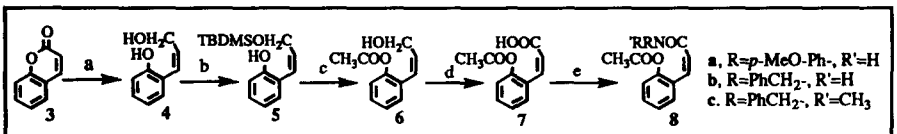
Scheme 1 A coumarin-based esterase-sensitive prodrug system.

To study the general applicability of this coumarin-based prodrug system, we chose three amines with different structural features as models of amine-containing drugs. These amines are *p*-methoxyaniline, benzylamine, and *N*-methyl benzylamine, representing aromatic amines, primary amines, and secondary amines. An acetyl trigger was chosen because it is known to be esterase-sensitive. Biological evaluation using porcine liver esterase shows that these prodrugs are indeed bioreversible in the presence of an esterase. However, the rate of release of the original amine depends on the structural features of the amine moiety.

Results and Discussion

Synthesis. The synthesis of all three model prodrugs started with commercially available coumarin. Due to the facile lactonization of coumarinic acid derivatives, it was not feasible to prepare the target compounds by direct acetylation of the phenol hydroxyl group followed by the attachment of the amine compounds to the carboxyl functional group. Instead, the side chain carboxyl group has to be converted to a protected hydroxyl group before the acetylation of the phenol hydroxyl group can be accomplished. Therefore, coumarin was reduced to the desired diol **4** using lithium aluminum hydride (LAH) at 0 °C in 15 min. Longer reaction time or higher reaction temperature results in reduction of the exocyclic double bond. The diol **4** was obtained by crystallization from the reaction mixture without the need for column chromatography. The allylic hydroxyl group of the diol **4** was then selectively protected as a silyl ether by reacting with *tert*-butyldimethyl silyl (TBDMS) chloride. The acetylation of the free phenol hydroxyl group was accomplished by reaction with acetic anhydride in the presence of triethyl amine (TEA) in a quantitative yield. The free allylic hydroxyl group after deprotection of the silyl group using acetic acid was converted to the carboxyl group in two steps. The oxidation to the aldehyde was accomplished using either pyridinium chlorochromate (PCC) or manganese dioxide in about 50% yield. Conversion of the aldehyde to the carboxylic acid **7** was accomplished in quantitative yield by oxidation with hydrogen peroxide in the presence of sodium chlorite under acidic conditions. The free acid was then coupled with the model amines using standard activation method using dicyclohexyl carbodiimide (DCC) as the activating

reagent in the presence of hydroxybenzotriazole (HOBt) and 4-dimethylaminopyridine (DMAP). All new compounds were fully characterized with ^{1}H NMR, MS, and elemental analysis.[32]



a. LAH, 0 °C, 15 min, 44%; b. TBDMSCl/THF, DMAP, 0 °C, 83% ; c. (i) Ac_2O, DMAP-TEA, 100%; (ii) H_2O-THF-HOAc (1:1:3), 100%; d. PCC and then H_2O_2, $NaClO_2$,10 °C, 50%; e. DCC/HOBt, DMAP, NRR', about 70%.

Scheme 2 Synthesis of the coumarin-based model prodrugs.

Esterase Kinetics. Although extensive work has been done studying the detailed mechanism and reaction kinetics of the lactonization of coumarinic acid system, most was not done under near physiological conditions. Furthermore, most of these kinetic studies were done with coumarinic acid itself rather than its ester or amide derivatives. Therefore, to study the feasibility of this coumarin-based prodrug system, it is necessary to study the lactonization kinetics under near physiological conditions using model amides (**8a–c**) of coumarinic acid. The key feature of the design is the release of the original drug moiety upon esterase catalyzed hydrolysis of the phenolic ester (Scheme 1). We chose to use porcine liver esterase (Sigma, E.C. 3.1.1.1) for the initial feasibility studies because it has been widely used in similar studies in the literature and the results correlate well with that of plasma studies.[2,13,14] HPLC was used for the reaction rate studies. The quantitative determination of the final product, coumarin **3**, was made by using a standard curve. Because of the facile cyclization of the potential intermediate, samples were taken out at different intervals and frozen immediately at –78 °C, which instantly stopped the reaction.

Table 1. Rates of the Appearance of Coumarin (**3**) from the Esterase-Triggered Lactonization of **8a–c**.

Model Prodrug	*k*obs (x10^3) (sec^{-1})	$t_{1/2}$ (Sec)
8a	7.01	98
8b	0.71	976
8c	0.37	1873

Results are average of duplicates

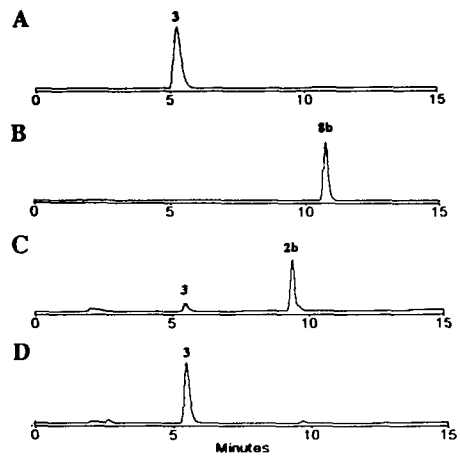


Figure 1. Typical HPLC chromatograms. Panel A: coumarin **3**; panel B: starting material **8b**; panel C: 1 min after the addition of esterase; panel D: 130 min after the addition of esterase.

When porcine liver esterase was incubated with the test compounds using a procedure similar to that published from Borchardt's group (pH 7.4, 37 °C, phosphate buffer 0.05 *M*),[13] all model prodrugs underwent a facile cleavage of the phenol ester. The esterase catalyzed hydrolysis of the phenol ester was very fast. For all three compounds **8a–c**, starting material almost completely disappeared within one minute. Instead, a new peak with a shorter retention time than the starting material appeared. This was presumably the intermediate (**2**, Scheme 1) after hydrolysis. A typical HPLC chromatogram (using **8b** as an example) is shown in Figure 1. Because of the rapid hydrolysis of the phenolic ester by porcine liver esterase, in essence the intramolecular cyclization reaction to form coumarin **3** is the rate limiting step for all three compounds (**8a–c**) studied. This is in direct contrast to the prodrug system using a "trimethyl lock" facilitated cyclization system, in which the porcine liver esterase catalyzed hydrolysis of the ester linkage is relatively slow and rate limiting.[13] The major difference between the coumarin-based system and the "trimethyl lock" facilitated cyclization system is the steric hindrance around the phenolic ester linkage. Because of the presence of essentially a *t*-butyl group adjacent to the phenolic hydroxyl group in the "trimethyl lock" facilitated cyclization system, esterase-catalyzed hydrolysis of this ester might be more difficult compared with coumarinic acid derivatives. Therefore, the coumarin-based prodrug system may be more susceptible toward esterase catalyzed hydrolysis because of the lack of steric hindrance.

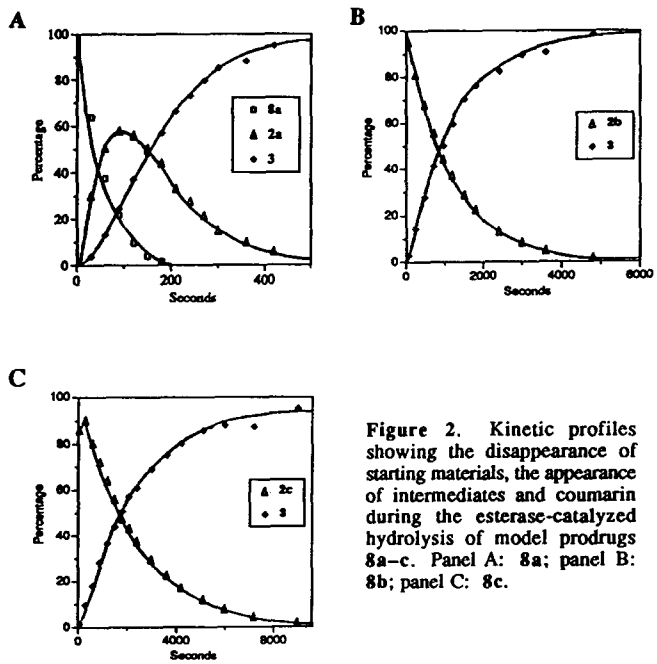


Figure 2. Kinetic profiles showing the disappearance of starting materials, the appearance of intermediates and coumarin during the esterase-catalyzed hydrolysis of model prodrugs **8a–c**. Panel A: **8a**; panel B: **8b**; panel C: **8c**.

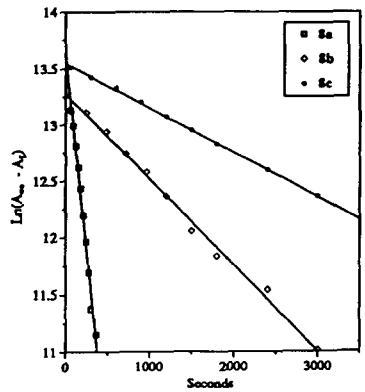
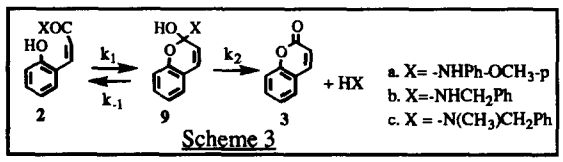


Figure 3. The pseudo-first-order kinetics of the esterase-triggered release of amines from model prodrugs **8a–c**.

The presumed intermediate (**2**, Scheme 1) then underwent lactonization at different rates depending on the structural features of the model amines (Table 1). For example, compound **8a** has a half life of about 98 s and compounds **8b** and **8c** have a half life of about 16 and 31 min, respectively (Table 1). The control reactions in the absence of porcine liver esterase showed less than 2% hydrolysis during the time course studied (60 min for **8a**, 120 min for **8b**, and 150 min for **8c**) (Table 1). Figures 2A–C show typical profiles for the appearance and disappearance of the intermediate and the appearance of coumarin during the esterase-catalyzed hydrolysis of **8a–**

c. Because the starting materials **8b–c** disappeared very rapidly during the time scale of the study, the concentration change of the starting material with time is not shown for these two compounds. The rate constants were calculated using the concentration increase of coumarin, the quantitation of which was carried out by using a standard curve.

There are very noticeable differences among all three compounds **8a–c** in their rates of amine release after the phenolic ester is cleaved by porcine liver esterase (Table 1). Coumarinic acid esters were known to lactonize at rates similar to that of the "trimethyl lock" system and yet all model prodrugs of the "trimethyl lock" system seem to lactonize at similar rates once the free phenolic hydroxyl group is generated.[12,13,17] However, extensive mechanistic studies have shown that the lactonization of coumarinic acid undergoes a unique change of rate determining step from low pH to high pH.[23–25] At low pH, the rate limiting step tends to be the nucleophilic attack of phenolic hydroxyl group on the side chain carboxyl group and at higher pH, the rate limiting step changes to the collapse of the tetrahedral intermediate leading to the lactone formation. Mechanistic studies using tetrahedral intermediates independently generated from other sources suggest that when the rate limiting step is the collapse of the tetrahedral intermediate, the partition between the elimination of a phenoxide ion to give the open-ring compound **2** and the elimination of the XH to give the lactone **3** depends partially on the leaving tendency of each leaving group (Scheme 3).[33] The difference in the rates (Table 1) of release of the model amines from **8a–c** is consistent with the mechanism of this lactonization.



Scheme 3

The slower lactonization of the *N*-methylbenzylamide (**8c**) and benzylamide (**8b**) seem to be due to the presence of the electron-donating alkyl functional group(s), which lowers the leaving tendency of the amino functional groups. On the other hand, anisidine (from **8a**) is expected to be a much better leaving group than its aliphatic counterparts due to the conjugation with an aromatic functional group. The different release rates between the model prodrugs of benzylamine **8b** and *N*-methylbenzylamine **8c** can also be accounted in electronic and/or steric terms because a methyl group can donate electron to the nitrogen and also introduce steric hindrance that hinders the attack of the amide by a nucleophile. Quantitative evaluation of the contribution of different structural features to the release rates is difficult to make with the limited number of compounds studied so far. It should be noted that the steric and electronic factor differences among these three amides **8a–c** did not seem to affect the porcine-liver esterase catalyzed cleavage of the phenolic ester linkage.

Conclusion

The chemical feasibility of a new coumarin-based, esterase-sensitive prodrug system is demonstrated with three model amines, representing aromatic amines, primary amines, and secondary amines. It was shown that porcine liver esterase can readily catalyze the hydrolysis of the phenol ester group triggering the facile lactonization process, which in turn leads to the rapid release of the model amines and the formation of coumarin. The rates of release of the amine compounds seem to depend on structural features of the amine moiety. However, the release

time ($t_{1/2}$) can be optimized by introducing functional groups with different electronic properties to the phenyl ring.[23,24] Due to the known toxicity profile of coumarin, no toxicity problem is expected with the introduction of this prodrug moiety. With the demonstrated chemical feasibility, others may apply the same strategy to the preparation of prodrugs of real drug entities for biological evaluations. Further structural studies are underway to elucidate quantitatively the influence of different structural factors on the release kinetics.

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32. For **8a**: ^{1}H NMR ($CDCl_3$): δ 2.35 (3 H, s, CH_3CO), 3.72 (3 H, s, CH_3O), 6.23 (1 H, d, J = 11.7 Hz, CHCO), 6.73 (2 H, d, J = 8.7 Hz, Ar-H), 6.83 (1 H, d, J = 12 Hz, CHCHCO), 7.21 (6 H, m, Ar-H), 7.93 (1 H, br, N-H); EIMS (relative intensity): 311 (M^+, 76), 252 (60), 147 (90), 123 (100), 108 (49), 43 (29); Anal. Calcd for $C_{18}H_{17}O_4N$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.18; H, 5.53; N, 4.64.
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