Catalytic Effect of Dipotassium Glycyrrhizinate on the Hydrolysis of Nonionic Ester Surfactants

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ABSTRACT: The family of dipotassium glycyrrhizinate (GK2) catalyzes the hydrolysis of nonionic ester surfactants (NES), such as hydrogenated castor oil with 60 oxyethylene units attached (HCO-60) and monostearate with 25 oxyethylene units attached (SA-25), even in the pH range of 3–6, where NES are generally stable in the absence of GK2. Long-chain fatty acids also accelerate the hydrolysis reaction. Analyses, based on circular dichroism spectroscopy, and empirical force field calculations suggest that a GK2 and an NES molecule form an energetically stable complex *via* hydrophobic interactions in which the ester carbonyl is in close contact with either one of the three different carboxyl groups in GK2, acting as an acid catalyst. *JAOCS 73,* 913–920 (1996).

KEY WORDS: Circular dichroic analysis, computer-aided molecular design, dipotassium glycyrrhizinate catalysis, hydrolysis of nonionic surfactants, hydrophobic interactions, long-chain fatty acid catalysis.

In the preparation of liquid pharmaceuticals, such as eye lotions and injectable formulations, dipotassium glycyrrhizinate (GK2), shown in Figure 1, serves as an anionic surfactant as well as an antiallergic and anti-inflammatory drug (1). Conversely, polyoxyethylene(60) hydrogenated castor oil (HCO-60) is used as an effective and safe solubilizing agent for oil-soluble ingredients, such as vitamins A, E, and K. Sometimes, however, precipitation occurs in injectable formulations that contain HCO-60 when stored for a long time and at high temperatures, and the precipitate was found to be 12-hydroxystearic acid (12-HSA), a major hydrolysis product of HCO-60 (2,3), although such hydrolysis was slow under neutral conditions. We also recently observed that precipitation takes place in eye lotions that contain both GK2 and HCO-60 under the conditions of long-time and high-temperature storage (3) and that the hydrolysis of HCO-60 or nonionic ester surfactant (NES) in general is accelerated in the presence of GK2. In view of widespread co-use of GK2 and HCO-60, it is important to investigate the more detailed features of GK2 effects. This paper describes how GK2 acts on the hydrolysis of HCO-60, based on reaction kinetics, circular dichroism (CD) spectroscopy, and computer-aided molecular design (CAMD) analysis. The compounds investigated are shown in Figure 1.

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EXPERIMENTAL PROCEDURES

Materials. GK2 and its family of monoammonium glycyrrhizinate, glycyrrhetic acid (GA), and stearyl glycyrrhetinate were obtained from Maruzen Seiyaku Co. (Hiroshima, Japan) and used as received. Reagent-grade NES—polyoxyethylene(*n*) hydrogenated castor oils (HCO-*n*), polyoxyethylene(20) sorbitan monooleate (SO-20), polyoxyethylene(20) sorbitan monostearate (SS-20), and polyoxyethylene(25) monostearate (SA-25)—were obtained from Nikko Chemicals Co. (Tokyo, Japan). 12-HSA, stearic acid, lauric acid, and nonadecanoic acid were obtained from Sigma (St. Louis, MO) and used as received. 9-Anthryldiazomethane was obtained from Funakoshi Co. (Tokyo, Japan) and used as received. Commercially available buffer reagents, including citric acid, were extra pure and used as received. Organic solvents were purified according to standard methods. Two different types of HCO-60 analogues, HCO-60N (normal sample) and HCO-60S (special sample), shown in Table 1, were specially prepared by Nikko Chemicals. HCO-60N was prepared by the ordinary method of reacting HCO with 60 moles of ethylene oxide and an alkaline catalyst. HCO-60S was prepared by a two-step process: HCO was reacted first

TABLE 1
Analytical Data of Two HCO-60 Samples[a]

Partial structures and properties	Mole % in HCO-60N	Mole % in HCO-60S
12-HSA (free 12-OH)	70.0	16.0
12-HSA [$-O(CH_2CH_2O)_nH$]	17.2	71.2
Stearic acid (SA)	11.8	11.8
Palmitic acid	1.0	1.0
CMC (mM)	0.06	0.08
γ (dyn/cm)[b]	43	39

[a]Obtained from Nikko Chemicals (Tokyo, Japan); HCO-60, polyoxyethylene(60) hydrogenated castor oil; 12-HSA, 12-hydroxystearic acid (Sigma, St. Louis, MO); CMC, critical micelle concentration.
[b]0.2% Aqueous solution.

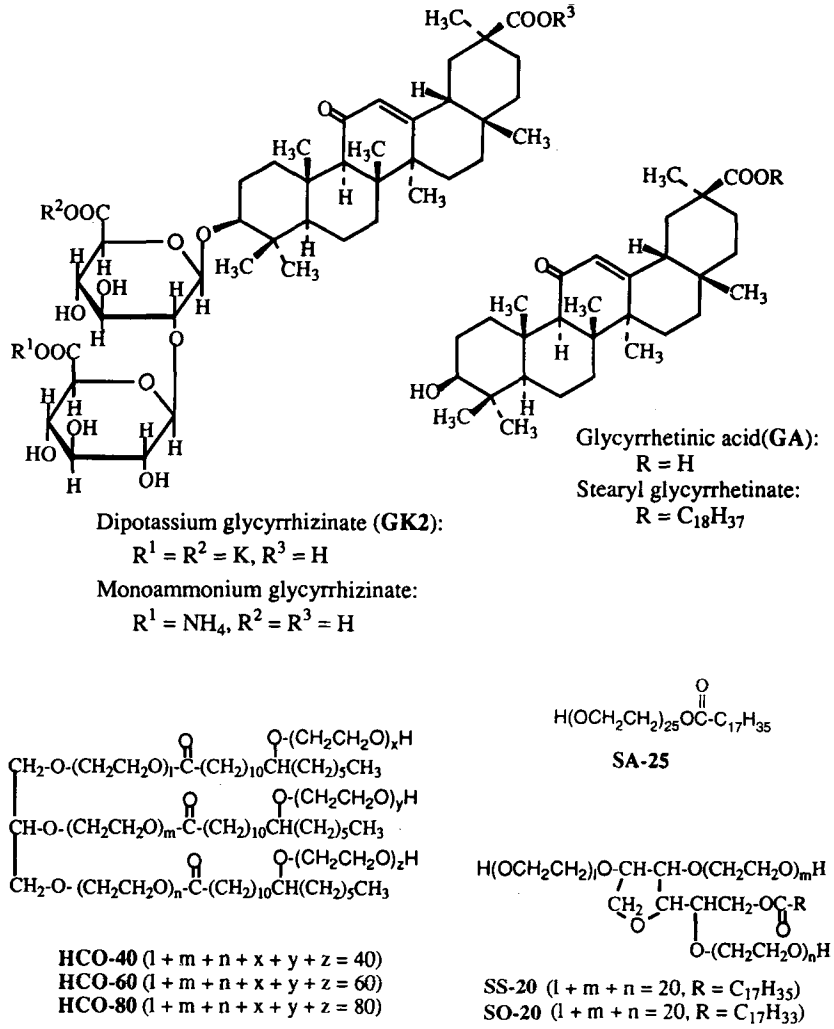


FIG. 1. Structures of GK2 families and nonionic ester surfactants. HCO-40, -60, -80, polyoxyethlene(40), -(60), -(80), respectively, hydrogenated castor oil; SO-10, polyoxyethylene(10) sorbitan monooleate; SA-25, polyethylene(25) monostearate; SS-10, polyoxyethylene glycol(10) sorbitan monostearate.

with 9 moles ethylene oxide by using an acid catalyst (BF_3/ether complex), then with 51 moles ethylene oxide by using an alkaline catalyst. The analytical data of the two samples are shown in Table 1. The 12-hydroxyl group largely remained free (70%) in HCO-60N, while it largely reacted with ethylene oxide (71.2 %) in HCO-60S (4).

Product analysis. An aqueous reaction mixture was extracted with chloroform. The organic layer was evaporated, and the residue was subjected to high-performance liquid chromatography (HPLC) on a reversed-phase (ODS) column. The obtained pure 12-HSA was identified by Fourier transform infrared, ^1H nuclear magnetic resonance (NMR), and mass spectra.

Kinetic procedures. Hydrolysis was performed as follows. A 0.1 M citrate-buffered reaction solution, containing appropriate concentrations of GK2 (1.11–4.4 mM) or fatty acid catalysts (1.11 mM) and surfactants (1.43 mM) to be hydrolyzed, was divided into the desired number of ampoules and incubated in a constant-temperature bath (70 ± 0.5°C). At appropriate time intervals, an ampoule was withdrawn from the bath for HPLC analysis as in the following procedures.

Preparation of standard solutions. (i) Two internal-standard methanol solutions, containing 2.5×10^{-3} and 2.5×10^{-4} M lauric acid (I-1 and I-2 solutions, respectively) were prepared for analysis of 12-HSA. Meanwhile, for stearic acid, *n*-nonadecanoic acid was employed instead of lauric acid as the internal standard. (ii) 2 mL of a standard methanol stock solution of 12-HSA (3.33×10^{-4} M) was mixed with 200 µL of I-1 solution and diluted tenfold to give the standard solution of 12-HSA.

Preparation of analytical solutions. Two mL of the reaction solution was mixed with 200 µL I-2 solution in a stoppered test tube. After addition of 5 mL chloroform, the tube was centrifuged under 2,000 × *g* for 5 min. The upper water layer was withdrawn by aspirator suction and discarded. The chloroform layer was then washed three times with 2 mL

water each, and centrifugation and aspirator suction were repeated. Following the complete removal of the water, 4 mL of the chloroform layer was withdrawn, and the solvent was removed by rotary-evaporation to dryness. The dried residue was dissolved with 2 mL methanol, which then served as the analytical solution.

Preparing 9-anthryldiazomethane solution. An acetone solution of 9-anthryldiazomethane (2.0 wt/vol %) was diluted tenfold with methanol. This solution should be used within 1 h because of instability of the diazomethane in solution.

Preparing analytical samples. The 9-anthryldiazomethane solution of 200 μL was added to 100 μL each of the above standard solution and analytical solution, and the mixture was stirred well and left for more than 2 h before HPLC analysis.

Fluorescence measurements. HPLC analyses were performed by using a temperature-controlled column (40°C) filled with Inertsil ODS-2, 5 μm, 4.6 mm × 250 mm (GL Science, Tokyo, Japan) with acetonitrile/water (90:10 vol/vol%) solvent. Fluorescence spectra were recorded on a Shimadzu RF-535 (Kytoto, Japan) fluorescence HPLC monitor with the use of excitation at 365 nm and emission at 416 nm.

CD spectral measurements. CD spectra of GK2 solutions were measured with a JASCO J-720 spectropolarimeter (Tokyo, Japan). The measurements were carried out at 20°C in 0.1 M citrate buffer that contained 1.1 mM GK2 and 1.43 mM NES as adopted for hydrolysis studies, unless otherwise specified.

CAMD calculations. Computational molecular mechanics calculations of stabilization energies of GK2/NES molecular complexes were performed with CHARMm Ver 22/QUANTA Ver 3.3 (Molecular Simulation Inc., San Diego, CA) on Indigo R-4000 (Silicon Graphics Corp., Mountain View, CA) hardware.

RESULTS AND DISCUSSION

Hydrolysis products. On prolonged heating of an aqueous solution of HCO-60, particularly in the presence of GK2, a precipitate appeared, which was identified as 12-HSA, as reported previously (3). Obviously, 12-HSA is a hydrolysis product, although its origin is not necessarily clear. It was suggested that 12-HSA is formed by the hydrolysis of ester chains that have a free 12-hydroxyl group left without reacting with ethylene oxide (3). It also could be possible, however, that 12-HSA is formed by the depolymerization of polyoxyethylene side chains. To answer this point, we obtained two different samples of HCO-60, i.e., HCO-60N and HCO-60S. These samples were hydrolyzed in the presence of GK2 under the same reaction conditions as described (Figs. 2 and 3) except that the temperature was kept at 60°C. The amount of 12-HSA produced from HCO-60S was about one-fourth of that from HCO-60N, indicating that the major part of 12-HSA comes from the ester chains with a free 12-hydroxyl group. The product from other NES is simply a fatty acid with no hydroxyl group.

Kinetics of GK2-catalyzed hydrolysis of NES: Analytical method. As mentioned above, the major product of hydrolysis of HCO-60 was 12-HSA. In previous reports, 12-HSA was analyzed by a calorimetric method (2) or by gas–liquid chromatography after methylation with diazomethane (3). In this study, we have explored a fluorescent HPLC method for the analysis of 12-HSA, which was derivatized with 9-anthryldiazomethane reagent prior to HPLC analysis, as shown in

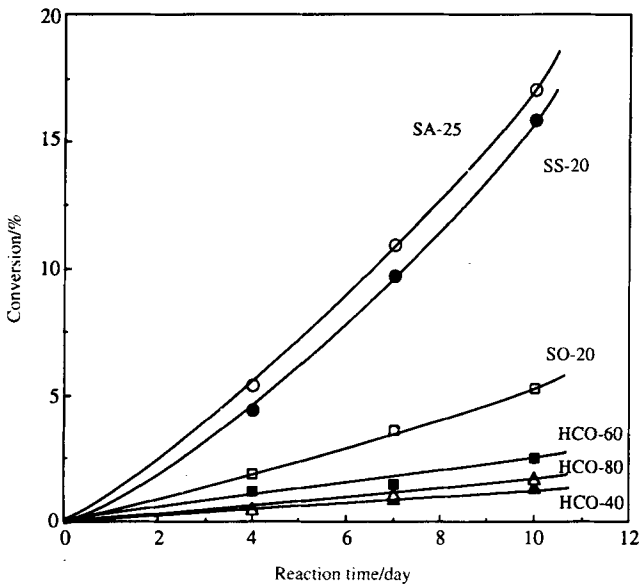


FIG. 2. GK2-catalyzed hydrolysis of nonionic ester surfactants (NES) in 0.1 M citrate buffer (pH 4.9) at 70°C: [NES] = 1.43 mM, [GK2] = 1.1 mM; SS-20, polyoxyethylene(20) sorbitan monostearate; SO-20, polyoxyethylene(20) sorbitan monooleate. See Figure 1 for other abbreviations.

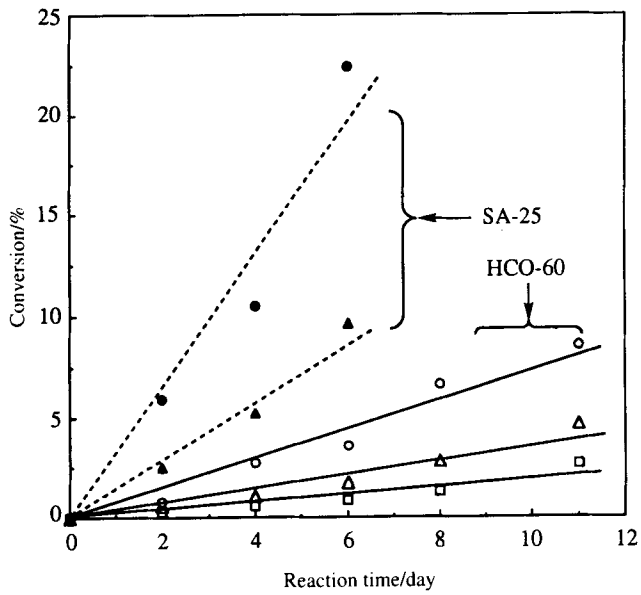
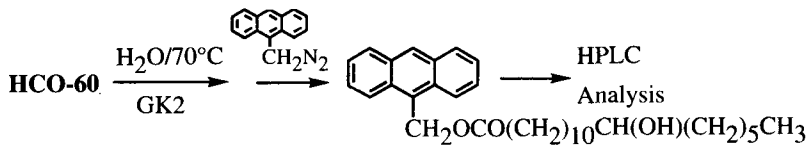


FIG. 3. Plots of % conversion vs. reaction time for the hydrolysis of HCO-60 and SA-25 in the presence of GK2 at pH 4.9: [HCO-60] = [SA-25] = 1.43 mM; [GK2] = 2.22, 1.11, 2.22, 1.11, 0.56 mM from the top; lines are arbitrary. See Figure 1 for abbreviations.



SCHEME 1

Scheme 1. The same method also could be applied for the quantitative analysis of stearic acid, produced from other NES, such as SA-25. The present method appears to be superior to the previous ones for ease and accuracy.

Effect of GK2 addition on the hydrolysis of different NES. In Figure 2 are the plots of conversion (%) vs. reaction time (day) for the hydrolysis of several NES at pH 4.9 and 70°C and in the presence of a given concentration of GK2; the conversion (%)/100 is defined as moles of 12-HSA per moles of NES used. The product is either 12-HSA or stearic acid, depending on the NES used. In the absence of GK2, practically no reaction was observed for each surfactant, indicating that the hydrolysis was catalyzed by GK2. Furthermore, Figure 2 shows that the rate tends to become larger autocatalytically with increasing reaction time. It is conceivable that the products 12-HSA and stearic acid also catalyze the hydrolysis. Also, the rates of hydrolysis of SA-25 and SS-10 are much larger than those of HCO-*n*, and monoammonium glycyrrhizinate is almost equally as active as GK2.

Effect of GK2 concentration. To confirm the above findings in more detail, the influence of altering GK2 concentrations was examined, and the results thus obtained are summarized in Figure 3, which shows that the slope of the line, i.e., the rate of reaction, increases with increasing concentration of GK2, roughly in a linear fashion for both SA-25 and HCO-60. More careful inspection of the data of HCO-60 again reveals that the conversion increases autocatalytically with increasing reaction time, suggesting that not only GK2 but also the product fatty acid is capable of acting as catalyst. The fact that SA-25 is much more active than HCO-60 implies that, under the present reaction conditions, SA-25 is much more susceptible to the catalysis of GK2 than is HCO-60 because, in the hydronium ion-catalyzed hydrolysis, the reactivity is not much different between NES (2,3,5).

Effect of the medium pH. The pH-conversion profiles for HCO-60 over the pH range of 3 to 8 are presented in Figure 4, which reveals that HCO-60 is quite stable in the absence of GK2 in a pH range of 4 to 7, even at 70°C, although the hydronium ion- and hydroxide ion-catalyzed hydrolyses become detectable at pH values below 3 and above 7, respectively. Conversely, a remarkable rate acceleration occurred in the presence of GK2, which became much more enhanced by lowering the pH. Evidently, GK2 acts as an acid catalyst. It is quite likely that such acid catalysis is caused by the carboxyl group(s) of GK2. Polar and hydrophilic acids, however, such as formic, acetic, and citric acids, employed as buffer reagents, showed no distinct acid catalysis. In view of the characteristic structural features of the GK2 molecule, it seems to be important that GK2 is a hydrophobic acid with a triterpenoid skeleton.

Hydrolysis catalyzed by fatty acids. The effect of the alkyl chainlength of carboxylic acids on acid catalysis was examined, and results are given in Figure 5. Carboxylic acids with long alkyl chains produced much larger amounts of 12-HSA than those with shorter alkyl chains. Thus hydrophobicity, or lipophilicity, is a useful criterion for the catalytic activity of a carboxylic acid. Furthermore, it was observed that glycyrrhetinic acid (GA) without a sugar moiety was almost equally as active as GK2, while the stearyl glycyrrhetinate ester showed no activity, indicating again the importance of the free carboxyl group for catalysis.

CD spectra. One of the means to study hydrophobic interactions is to examine CD spectra. GK2 is an optically active compound with an enone chromophore. Thus it is a suitable molecule for that purpose.

GK2 concentration dependency of CD spectra. GK2 is a surfactant, and its critical micelle concentration (CMC) value is 1.1 ± 0.3 mM at pH 4.9 in citrate buffer. Figure 6 shows the CD spectra taken at several concentrations of GK2. CD spectra of GK2 depended highly on its concentrations. Below the CMC, there are no clear absorption maxima due to the Cotton effect (6), while, above the CMC, there appear two dis-

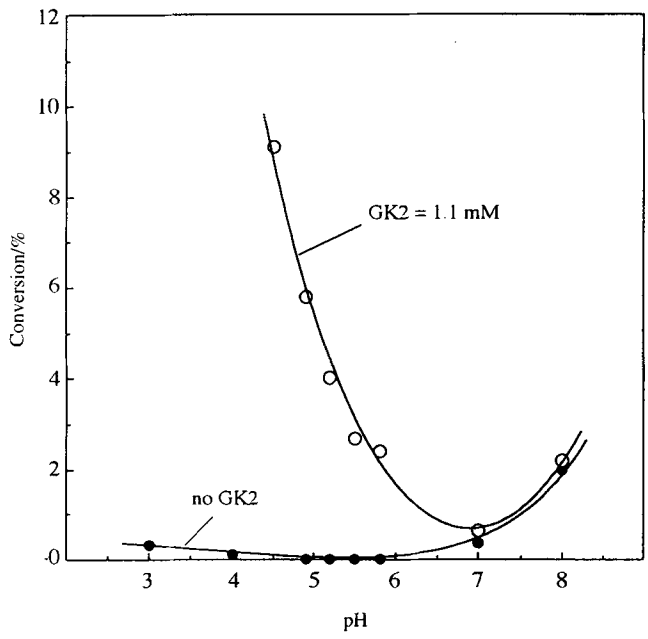


FIG. 4. Effect of medium pH on the GK2-catalyzed hydrolysis of HCO-60 for 2 wk. See Figure 1 for abbreviations.

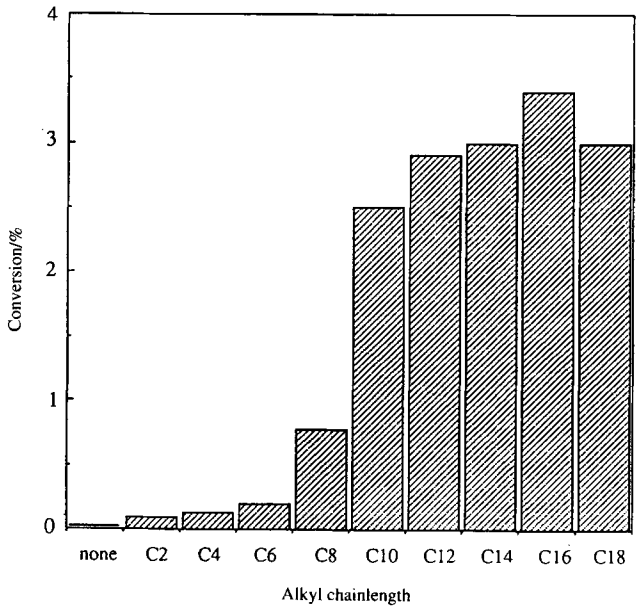


FIG. 5. Effect of alkyl chainlength of fatty acids on their catalytic activities for the hydrolysis of HCO-60 for 1 wk at pH 4.9: [fatty acid] = 1.1 mM. See Figure 1 for abbreviation.

cernible absorption bands at λ_{max} 281 ± 0.5 and 340 ± 0.5 nm with a 1.1 mM solution of GK2. These can be ascribed to the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions due to the enone chromophore, respectively. The former absorption band shifts further to 290 nm at 5.55 mM solution. It is obvious that the spectra below and above the CMC correspond to those of the monomer state and the micellar aggregate state, respectively, and the microenvironment around the enone chromophore is widely different between these two states.

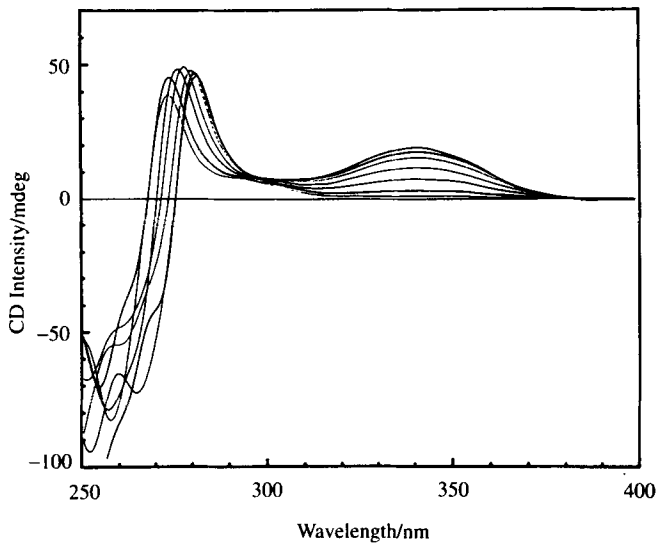


FIG. 7. Changes in CD spectra of 1.11 mM GK2 solutions with varying concentrations of SA-25: [SA-25] = 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 mM from the top. See Figures 1 and 6 for abbreviations.

Effect of NES concentrations on CD spectra of GK2. Intensity at 340 nm decreased with increasing concentrations of NES for both SA-25 and HCO-60, as typically shown in Figures 7 and 8. It is likely that, with increasing NES concentration, the initial micellar aggregates of GK2 are dispersed in the NES micelles in monomeric state where each monomer exists in a more hydrophobic microenvironment, thereby causing a lower intensity at 340 nm as well as a shorter wavelength shift of the 280 nm band.

Effect of tetrahydrofuran (THF) cosolvent on CD spectra of GK2. As shown in Figure 9, the CD intensity at 340 nm decreased with increasing fractions of THF. Here it is almost

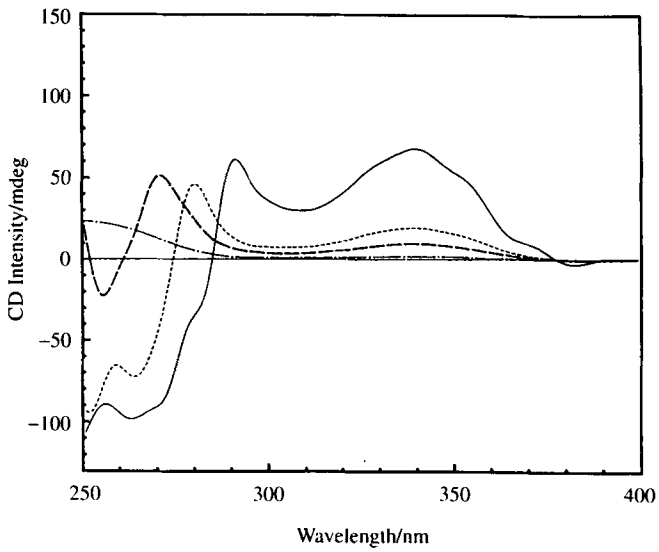


FIG. 6. Circular dichroism (CD) spectra of different concentrations of GK2 in 0.1 M citrate buffer of pH 4.9 at 20°C: [GK2] = 0.11, 0.56, 1.11, 5.55 mM from the top. See Figure 1 for other abbreviation.

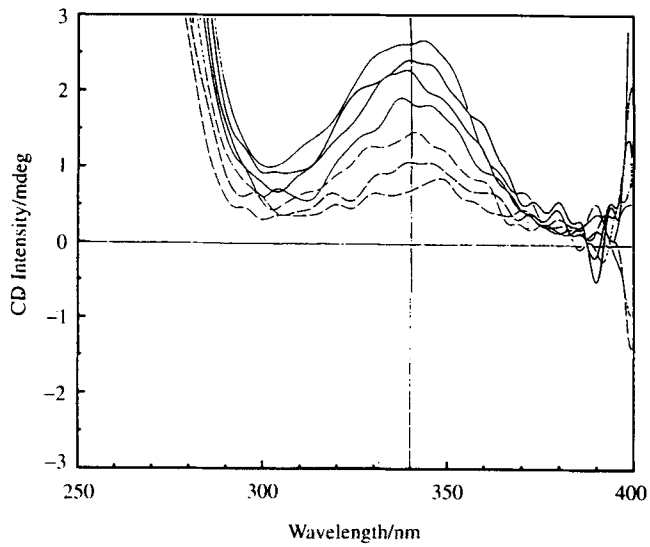


FIG. 8. Changes in CD spectra of 0.1 mM GK2 solutions at varying concentrations of HCO-60: [HCO-60] = 0, 0.15, 0.30, 0.60, 1.2, 2.4, 4.8 mM from the top. See Figures 1 and 6 for abbreviations.

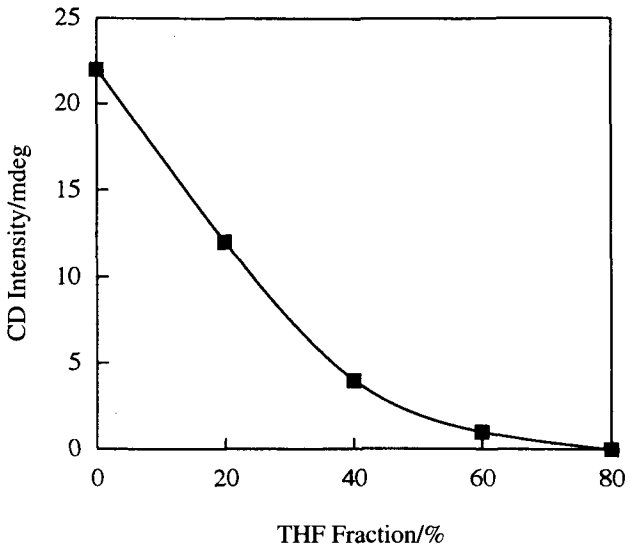


FIG. 9. Plot of CD intensity of tetrahydrofuran (THF)/water (vol/vol) binary solutions containing 1.1 mM GK2 as a function of the THF fraction. See Figures 1 and 6 for other abbreviations.

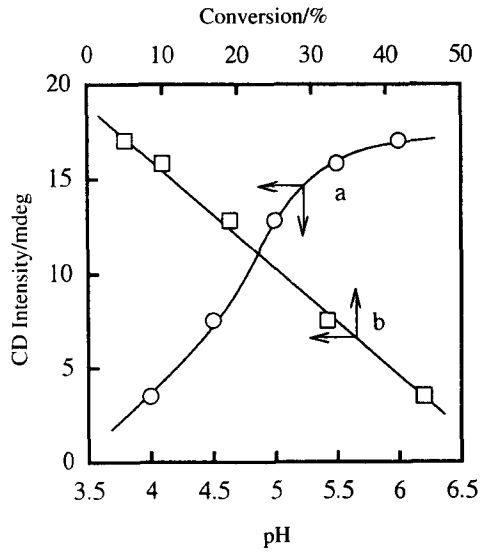


FIG. 10. Influence of changing pH on (a) CD intensity and (b) % hydrolysis for 2 wk with a 1.1 mM GK2/1.43 mM SA-25 combination. See Figures 1 and 6 for abbreviations.

certain that GK2, dissolved in THF as the monomer, is surrounded with hydrophobic THF solvents.

The above results therefore indicate that the absorption of GK2 at 340 nm is a sensitive measure to ascertain the microenvironment around the enone chromophore. Thus the effect of added THF shows that CD intensity at 340 nm becomes lower as the solvent becomes more hydrophobic. The reason for the low intensity observed below the CMC in aqueous solution seems to be due to the orientation of GK2 in the monomer dispersion, where the hydrophilic sugar moiety is dissolved in the water phase at the water–air interface, while the enone chromophore, buried in a hydrophobic triterpenoid skeleton, protrudes partly into the highly hydrophobic air phase. Conversely, the exact reason why the GK2 micelle exhibits a large absorption at 340 nm is difficult to explain presently because of limited information on the structure of GK2 micelles (7). Such a large absorption seems to suggest that the microenvironment of the enone chromophore in the GK2 micelle is highly polar or structured by hydrogen-bonding networks in the micellar core that involve the carboxyl groups on the triterpenoid terminals. In accord with this inference, CD absorption intensity of the modified GK2, with the triterpenoid carboxyl group being methyl-esterified (the synthetic procedure will be described in detail in the succeeding paper), was one-sixth smaller than that of GK2; namely, the micellar interior of the esterified GK2 is relatively less polar compared with the interior of the original GK2 micelle.

Effect of pH on the CD spectra of GK2. CD intensity of GK2 at 340 nm is remarkably pH dependent under kinetic conditions with the presence of SA-25 micelles, as indicated in Figure 10, where line a shows that the pH dependency was sigmoidal, as if to show the dissociation of a carboxyl group. There are three carboxyl groups in the GK2 molecule with pKa_1 3.3, pKa_2 4.7, and pKa_3 8.5, respectively. The first two are those of the sugar parts, and the third is that of the triterpenoid part.

The distinctly observed inflection point clearly indicates that the sigmoidal curve corresponds to the dissociation of one of the two sugar-carboxyl groups. Thus the ionization state of GK2 at lower pH values (below 4) is such as one sugar-carboxyl group is dissociated and the other two carboxyl groups are undissociated, while at higher pH values (above 7), the two sugar carboxyl groups are dissociated and the triterpenoid carboxyl group is undissociated. Here it is important to note that the GK2 molecule in the former ionization state is located in the more hydrophobic environment with a low CD intensity at 340 nm, while that of the latter ionization state is located in the more hydrophilic environment with a high CD intensity. Interestingly, Figure 10 (line b) also shows that there is a linear relationship between the conversion and the CD intensity, i.e., more conversion with lower intensity.

The above interpretation of the pH effect on the CD intensity is further supported by the pH effect on the CD spectra of GA in the SA-25 micelle. Because GA lacks a sugar part and has only one carboxyl group in the triterpenoid part, GA is much more hydrophobic than GK2, and its CD spectra would be indifferent to the change of pH. As expected, Figure 11 indicates that there is essentially no pH effect on the CD spectra of GA with pH values between 4–6 and with a low CD intensity at 340 nm.

Mechanism of acid catalysis: stabilization energies of GK2/NES complexes calculated by the CAMD system. The data shown in Figures 2, 3, and 4 indicate that GK2 is an active catalyst for the hydrolysis of NES, and the data of Figure 5 show that such acid catalysis is due to the carboxyl groups attached to lipophilic molecules. Further support is the fact that GA, which has a carboxyl group, is active, but its

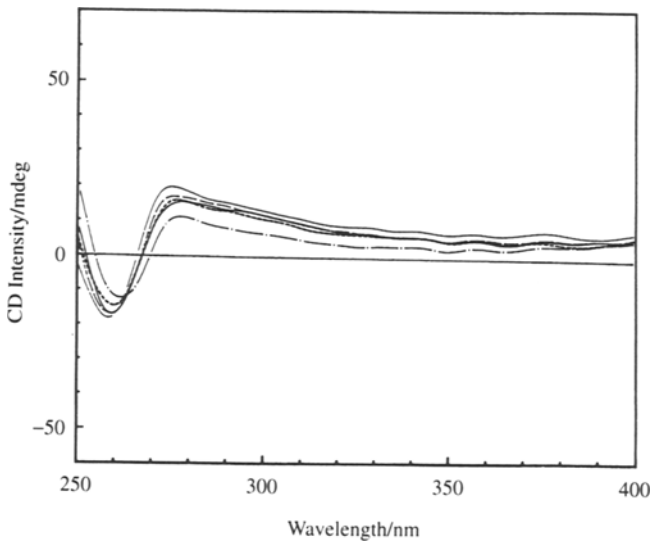


FIG. 11. Influence of pH change from 4 to 6 on CD spectral intensity for a 1.1 mM GA/1.43 mM SA-25 combination. See Figures 1 and 6 for abbreviations. See Figures 1 and 6 for abbreviations.

stearyl ester is inactive. It is likely that a carboxyl group acts as a general acid catalyst *via* hydrogen bonding to the ester carbonyl oxygen in the rate-determining nucleophilic attack to carbonyl carbon (8–10), where the attacking nucleophile is water or a carboxylate anion, although the more detailed mechanism is difficult to describe at present. In this paper, focus is placed on the fact that only lipophilic acids are active.

For any mechanism of the carboxylic acid catalysis, it is necessary to bring the reaction partners together in close proximity and in the right orientation for hydrogen bonding between the carboxyl group and the ester carbonyl oxygen. Figure 12 shows several possibilities for such hydrogen bondings at pH 4.9 by taking a single-chain SA-25 as the representative of NES substrates for simplicity. In the micelles of SA-25, the polyoxyethylene and hydrocarbon chains form the hydrophilic and hydrophobic domains, respectively. As discussed above, the GK2 molecule exists largely as a monoanionic state at pH 4.9, in which one of the two carboxyl groups of the sugar moiety and that of the triterpenoid moiety are undissociated and capable of forming hydrogen bonding with the ester carbonyl oxygen. Figure 12 shows four types of hydrogen bonding complexes of GK2, which is oriented in two opposite directions. The orientation in the complexes of **a** and **b** is such that the hydrophilic sugar parts are directed to the hydrophilic domain, while the hydrophobic triterpenoid part is directed to the hydrophobic domain of the SA-25 micelle. Conversely, the orientation in the complexes of **c** and **d** is the reversal of that in **a** and **b**. In **a** and **b**, the GK2 and SA-25 molecules can enjoy mutual favorable interactions, i.e., the hydrophobic bonding between the hydrocarbon chain and the triterpenoid part as well as the hydrogen bonding between the polyoxyethylene chain and the hydroxy groups of the sugar parts, including water molecules. On the other hand, in **c** and **d**, particularly in **c** the hydrophilic sugar parts are buried in

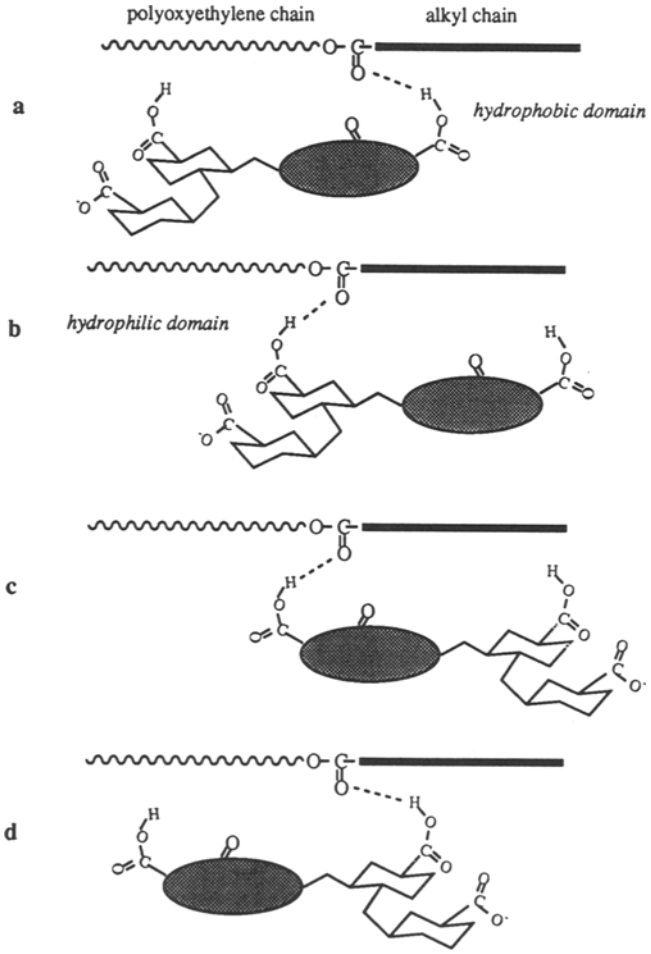


FIG. 12. Modes of complexation between a GK2 molecule and a SA-25 molecule. See Figure 1 for abbreviations.

the hydrophobic domain, which should be energetically unfavorable. As expected, empirical force field calculations with the CAMD system disclosed that the stabilization energy for the complexation is in the order of **b** ≥ **a** > **d** ≫ **c**, as summarized in Table 2. As discussed above, the pH-dependent change of CD spectra (Fig. 10) suggests that the dianion of GK2 in the SA-25 micelle is located at a more hydrophilic domain and moves to a more hydrophobic domain when protonated to the monoanion. Thus **b** is a good model of the monoanion complex, while **a** is rather suitable for the dianion complex.

TABLE 2
Stabilization Energy in Complexation of GK2 with SA-25

SA-25/GK2	Complex[a]	Stabilization energy (SE)/kcal/mol[b]
1:1	**a**	36.8
1:1	**b**	37.4
1:1	**c**	15.2
1:1	**d**	31.1

[a]See Figure 12.
[b]SE = Energy of the complex—sum of the energies of individual components, calculated by computer-aided molecular design system. See Figure 1 for other abbreviation.

The fact that only a hydrophobic acid is active implies that the formation of a tight complex with a high stabilization energy is critical for effective catalysis. For elucidation of the more detailed mechanism, it is necessary to specify the roles of three carboxyl groups of GK2 in catalysis. Their selective modification is currently in progress.

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