Effect of Glycyrrhizic Acid on Lappaconitine Phototransformation

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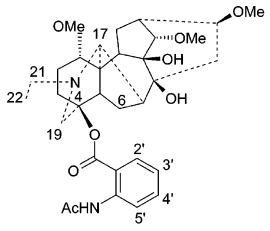
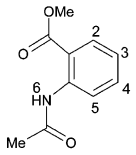
^{1}H NMR and CIDNP methods were used to demonstrate that triterpene glycoside (glycyrrhizic acid, GA) can substantially change the efficiency and direction of phototransformation of alkaloid lappaconitine (LA) due to both its solubilization in GA micelles and protonation of LA amine nitrogen in water−alcohol solutions. The LA solubilization in the GA micelle suppresses the process of deacylation.

1. Introduction

In evolutionarily advanced plants, alkaloids play a particular role fulfilling a function of plant protection against viruses, funguses, and microorganisms. Alkaloids both in pure form and resulting from various chemical transformations are widely used to produce physiologically active substances.[1] Lappaconitine—an alkaloid isolated from aconite—demonstrates bradycardic and hypotensive activity.[2−4] In pharmacology it is employed as the hydrobromide salt of lappaconitine called "Alapinine". Chemically, lappaconitine is an ester of triatomic alcohol lappaconin and *N*-acetyl-anthranilic acid. The structure of the LA molecule predetermines its photochemical lability which is not only of fundamental interest but also affects the application of LA as a drug.[5,6]

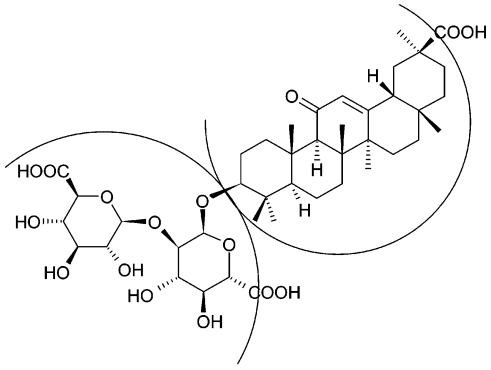


Lappaconitine (LA)



Methyl ester of N-acetyl-anthranilic acid (MA)

The present paper uses NMR and CIDNP methods to study the influence of triterpene glycoside−glycyrrhizic acid (GA) on the lappaconitine photolysis in solution. Interest to these studies was inspired by the finding of a considerable enhancement of the therapeutic activity of lappaconitine in the presence of GA accompanied by a decrease in the toxicity of preparation.[7] It is worth noting that similar effects were also observed for a series of other drugs.[8,9] In the pharmacological literature the effect of GA is usually assigned to formation of the complexes with drug molecules.[10,11] However, in the literature there are only assumptions on the structure of these complexes. It was assumed that at low concentrations GA could form cyclic dimer structures that contain hydrophobic cavities and are stabilized due to intermolecular hydrogen bonds.[12] The existence of cavities allows formation of complexes of the "host−guest" type. Most known examples of such aggregates are cyclodextrine inclusion complexes.[13] On the other hand, the presence of both hydrophobic (triterpene fragment) and hydrophilic (two glucoronic residue) moieties in the GA molecule allows one to assume that GA and its derivatives might form micelles in aqueous solutions.[14]



Glycyrrhizinic acid (GA)

Recently, our efforts were directed to studies arising from the interaction between GA and some biologically active compounds: nifedipine,[15] lappaconitine,[5] carotenoids,[16,17] and methyl ester of *N*-acetyl-anthranilic acid[18] in water−alcohol solutions. These studies suggested formation of two types of GA aggregates—a complex and a micelle. Small aggregates of GA and drug molecules with a stoichiometry of 2:1 were observed at low GA concentrations (10^{-3}−10^{-5} M).[5] At high concentrations ($\geq 10^{-3}$ M) GA forms large micellar-like associates.[18] In particular, evidence of micelle formation was obtained by NMR relaxation techniques (measurement of spin−spin relaxation time T_2) for complexes of GA and *N*-acetyl-anthranilic acid methyl ester (MA).[18]

The goal of the present work is to study LA solubilization in GA and the effect of GA on various stages of LA photodecomposition. In addition to the chemically induced dynamic nuclear polarization (CIDNP) method,[19−22] we used ^{1}H NMR (line width analysis and spin−spin relaxation time T_2 measurement).

2. Experimental Section

2.1. Chemicals. Deuterated solvents (D_2O (99.9% D), CD_3OD (99.5% D), Aldrich) were used as supplied. Glycyrrhizic acid, lappaconitine, and the methyl ester of *N*-acetyl-anthranilic acid were kindly donated by Prof. N. F. Salakhutdinov (NIOC,

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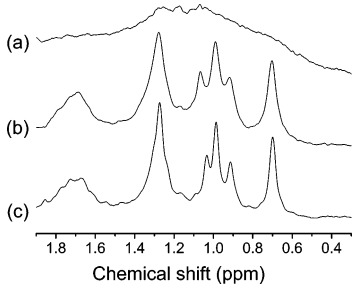


Figure 1. ^{1}H NMR spectra of glycyrrhizic acid methyl groups in the presence of 1 mM LA in 20% CD_3OD at different GA concentrations: (a) 1.5, (b) 1, and (c) 0.5 mM.

Novosibirsk). To prepare the basic medium (pH = 10) we used dry recrystallized NaOH. A mixture of D_2O with CD_3OD in a 1:1 and 4:1 ratio was employed to study the associations of GA and its effect on LA photoreaction. Solutions were deaerated by Ar bubbling.

2.2. Experimental Methods and Equipment. All NMR experiments (including photo-CIDNP and measurement of T_2 relaxation) were performed using an NMR DPX200 Bruker spectrometer equipped with a photoprobe and temperature control. An excimer laser Lambda Physik EMG101 (XeCl, 308 nm, 100 mJ) was used as a light source. All relaxation time measurements were carried out at 25 °C. Photo-CIDNP experiments were performed at room temperature. Photo-CIDNP was recorded using both time-resolved[23] and quasi-stationary variants[24] of pulse sequences. T_2 relaxation was measured by means of the Carr−Purcell−Meiboom−Gill sequence: $p(90°) - (\tau - p(180°) - \tau)_n -$ acquisition,[25] where $\tau = 2$ ms and n was varied from 0 to 4028.

2.3. Sample Preparation. LA concentration was either 0.5 or 1 mM in all experiments. GA concentration was varied from 0.5 to 5 mM. GA solutions were prepared according to the following scheme. First, GA was dissolved together with LA in CD_3OD, and then D_2O was added to the volume ratio of either 1:1 or 1:4. To equilibrate the sample, the GA solutions were heated to 50 °C and mixed for 2 h.

3. Results and Discussion

3.1. Study of GA Association in Water−Alcohol Solutions in the Presence of LA. To study the association of GA in water−alcohol solutions we analyzed NMR spectra of GA solution and measured the T_2 relaxation time of the methyl groups of GA. Figure 1 shows the broadening of NMR lines of methyl groups of GA with an increase in GA concentration.

The line broadening is assigned to an increase in the spin−spin relaxation rate ($1/T_2$) of the given protons. It is known that in liquid a change in the nuclear T_2 relaxation rate depends on the change in the rotational correlation time of the particle possessing the nuclei under study.[26] The rotational correlation time depends, in turn, on the particle size, which can vary substantially upon association.[27] Recently, we studied the dependence of the transversal relaxation rate ($1/T_2$) of GA methyl protons on its concentration.[18] It was established that the time dependence of the echo signal intensity is not described by a single exponent even at low GA concentrations (0.5 mM).[18] However, it was well approximated by a biexponential function

$$A(t) = P_1 \cdot \exp(-t/T_{21}) + P_2 \cdot \exp(-t/T_{22}) \qquad (1)$$

In the present work similar dependences of the transversal relaxation rate ($1/T_2$) of the methyl protons of GA on its concentration were measured in the presence of LA to prove formation of micelles. We found that time dependences of the echo signal intensity (Figure 2) could also be well described by function 1.

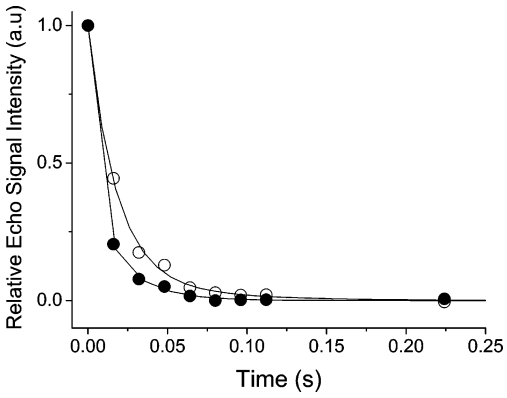


Figure 2. Changes in the echo signal intensity of GA methyl protons in the presence of LA in 20% CD_3OD as a function of time at different GA concentrations: (○) 0.5 and (●) 2 mM. Solid lines correspond to best-fitting curves.

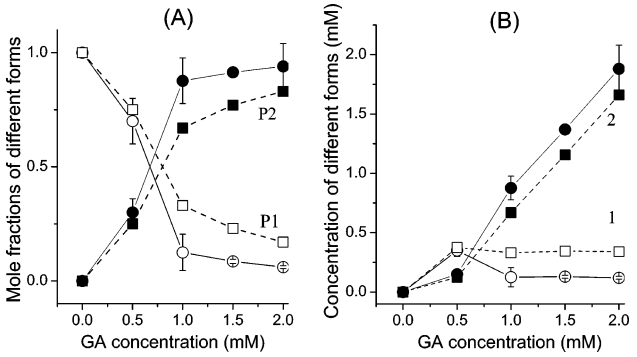


Figure 3. (A) Dependence of the relative premicellar P_1 (○) and micellar P_2 (●) fractions in the transversal relaxation function 1 on the GA concentration in the presence of LA. (B) Changes in the concentration of GA in premicellar 1 (○) and micellar 2 (●) forms as a function of GA concentration in the presence of LA. Squares and dotted lines have the same dependences in the presence of MA.[18] All data correspond to 20% CD_3OD.

In the literature there are many examples[28,29] of biexponential relaxation observed in investigations of the association of monomeric compounds into micelles, clusters of liquid crystals, etc. One of the best-known explanations of the observed phenomena is based on the difference in relaxation rates of protons of the same kind located in either the associate or its periphery.[29] Another explanation could be based on the difference in relaxation rates of the protons of the same kind located in either the associate or solution where the molecule is in the monomeric state. In this case, the relaxation times T_{21} and T_{22} refer to protons in solution and associate and P_1 and P_2 are the probabilities to find the molecule in the monomer or associated states, respectively. However, the latter is probable only in the case of slow exchange between the forms.[30] Under our experimental conditions the fast part of the time dependence of the echo signal corresponds to relaxation times below 10 ms. Therefore, assuming that a characteristic time of the exchange of GA molecules between the associate and solution corresponds to long times (the corresponding rate being less than 10^{-3} s^{-1}), we determined the P_1 and P_2 ratio for each GA concentration in the presence of LA. In this case, taking into account the dependence of correlation times on particle size, the long relaxation times were assigned to molecules in solution

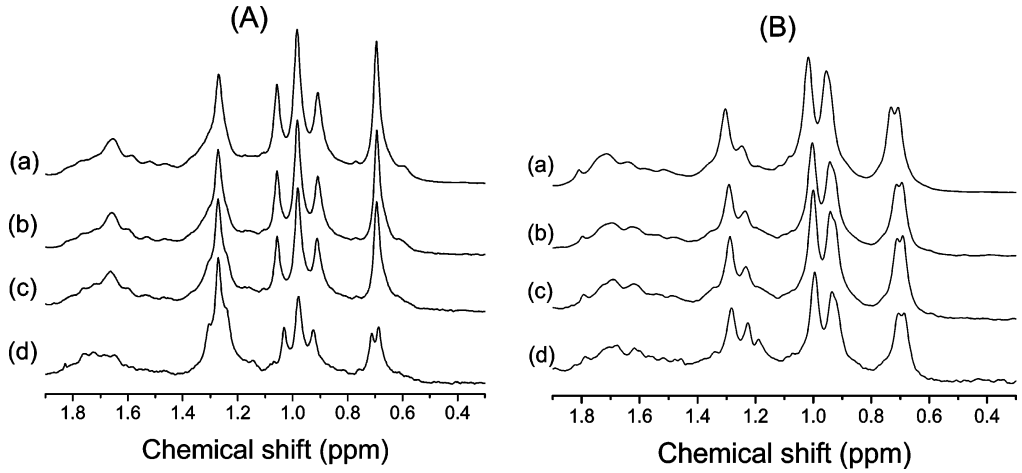


Figure 4. (A) ^{1}H NMR spectra of GA methyl groups at different concentrations of GA in 20% CD_3OD at pH = 3.5: (a) 5, (b) 1.5, (c) 1, and (d) 0.5 mM. (B) ^{1}H NMR spectra of GA methyl groups at different concentrations of GA at pH = 10: (a) 30, (b) 1.5, (c) 1, and (d) 0.5 mM.

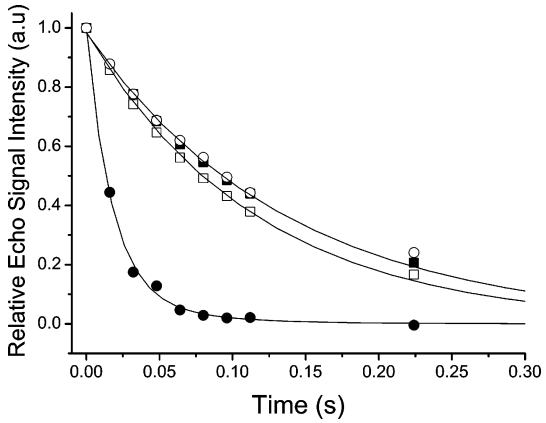


Figure 5. Changes in the echo signal intensity of methyl protons of GA at as a function of time in different conditions: (●) [GA] = 1 mM, [LA] = 1 mM in 20% CD_3OD; (○) [GA] = 2 mM, [LA] = 1 mM in 50% CD_3OD; and (■) [GA] = 2 mM, (□) [GA] = 30 mM without LA at pH = 10. Solid lines correspond to best-fitting curves.

and the shorter ones to the associates. This allows one to determine a change in the GA molecule distribution between the monomeric and associated forms depending on GA concentration.

Figure 3A shows the P_1 and P_2 dependences on GA concentration in the presence of LA. Using the concentration dependences of P_1 and P_2 we can readily obtain the dependence of GA concentration in various forms on the initial GA concentration (Figure 3B). For comparison, the same dependences in the presence of MA[18] are presented at Figure 3. The monomer concentration is $[GA_{Mon}] = P_1[GA]$, and that of the associate is $[GA_{Ass}] = P_2[GA]/M$, where M is the association number. It is worth noting that the plots obtained in the present work correlate well with those obtained in other studies.[18,29,31,32] Thus, we can make the conclusion about micelles formation under our experimental conditions (20% CD_3OD). On the other hand, in 50% CD_3OD we have not detected any concentration dependence of line width (Figure 4A) or relaxation times (Figure 5) in the same GA concentration range (0.2−5 mM).

Similar measurements were also made in basic media at pH = 10 in 20% CD_3OD (Figures 4B and 5). These experiments show no substantial effect of GA concentration on line broadening or relaxation rates of given protons. This suggests that the micelles of glycyrrhizic acid are not formed in basic solution as well as at the high alcohol content (50% or more). In the case of basic solution, the reason for destruction of GA

TABLE 1: T_2 Relaxation Time Values (T_{21} and T_{22}) from Eq 1 at pH = 3.5 and T_2 from Eq 2 at pH = 10 of the GA Methyl Protons in 20% CD_3OD at Different GA Concentrations

	relaxation times, ms		
[GA], mM	T_2	T_{21}	T_{22}
0.5		45 ± 4	9.6 ± 0.2
1		51 ± 17	10 ± 0.4
1.5		151 ± 60	9.6 ± 0.7
2	137 ± 2	164 ± 70	9.5 ± 0.3
5	141 ± 2		
10	139 ± 3		
15	142 ± 3		
30	117 ± 2		

associates might be due to destruction of hydrogen bonds between carbonyl groups.

As an illustration, Figure 5 shows the decay of an echo signal in acid media (pH = 3.4,), basic media (pH = 10), and 50% CD_3OD. Note that the curve at pH = 10 almost coincides with the one in 50% CD_3OD and is well described by the monoexponential function (instead of the biexponential function which describes dependences at pH = 3.4)

$$A(t) = P_0 \cdot \exp(-t/T_2) \qquad (2)$$

Data on T_2 relaxation times of the methyl protons of the molecule GA for various pH values are summarized in Table 1.

These results allow us to conclude that micelle formation occurs in 20% methanol solution only at neutral or acidic pH in the range of GA concentration exceeding 1 mM. Then in basic media as well as at high methanol content the GA molecules exist only in the premicellar form. The absence of micelle in 50% alcohol might be due to specific solvation of GA molecules by methanol.

Finally, we are going to discuss the size of the GA micelle. In the literature[33] there are several examples of the dependences of the mole fraction of molecules in the micellar state on their overall concentration for various association numbers at the same critical concentration of micelle formation (cmc). For small association numbers these dependences are smooth without sharp changes in the mole fraction in the cmc region. An increase in the association numbers causes a drastic change in the mole fraction within the cmc region. The dependence of the mole fraction of GA molecules in the micellar state (Figure 3A) is not characterized by sharp changes in the mole fraction

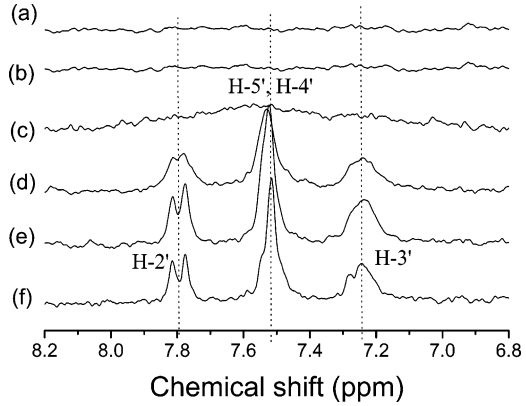


Figure 6. Partial ^{1}H NMR spectra of aromatic protons anthranilic fragment of 1 mM LA at different GA concentrations in 20% CD_3OD: (a) 5, (b) 2, (c) 1.5 mM, (d) 1, (e) 0.5, and (f) 0 mM.

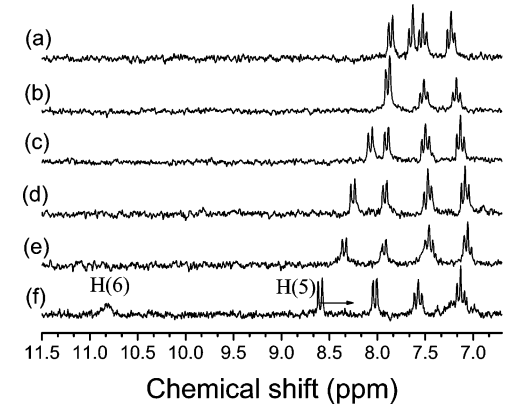
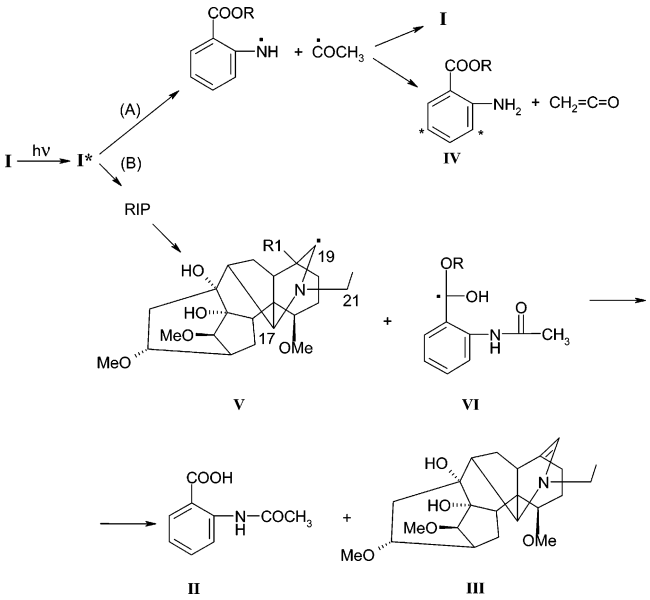


Figure 7. ^{1}H NMR spectra of 1 mM MA in CD_3OD/D_2O mixture: (a) 80% D_2O, (b) 50% D_2O, (c) 30% D_2O, (d) 10% D_2O, (e) 0% D_2O, and (f) CD_3CN.

SCHEME 1: Phototransformation of Lappaconitine



in the region of 0.5−1 mM. This fact indicates that in the case of GA we are dealing with small aggregation numbers ($M < 10$).

3.2. Study of GA Effect on Lappaconitine Phototransformation. The next step of the present study is the investigation of the influence of GA in micellar and premicellar forms on the LA phototransformation. Figure 6 illustrates the influence of GA on the ^{1}H NMR spectrum of aromatic protons of LA.

By increasing GA concentrations we clearly observe a substantial broadening of LA lines. The changes observed indicate an increase in the relaxation rate of LA aromatic protons with increasing GA concentrations. A possible reason for this effect might be penetration of the lappaconitine molecule inside the GA micelle. In this case, solubilization of the LA molecule in the micelle displaces, on one hand, water from its nearest environment of LA and, on the other hand, leads to an increase in the rotational correlation time of the LA molecule compared to that in homogeneous solution.

As mentioned above, earlier we demonstrated the effect of micelle formation on the photodecomposition of *N*-acetyl-anthranilic acid methyl ester (MA).[18] The monomolecular process of diacylation is the main reaction for MA photolysis. It was established that the intermolecular hydrogen bonds of MA carbonyl groups with water protons are destroyed inside GA micelles. This, in turn, leads to reduction of the intramolecular hydrogen bond in the MA molecule (see the H(6) line in Figure 7) and a decrease in the efficiency of the photoinitiated reaction.

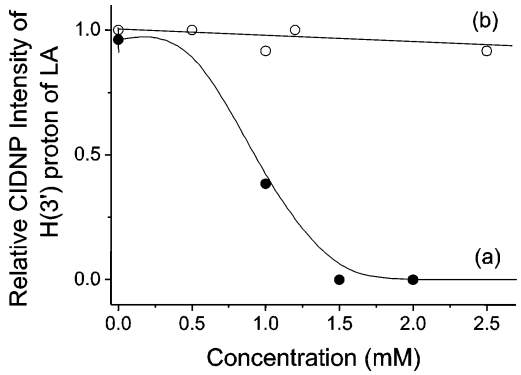


Figure 8. Dependence of the relative CIDNP intensity of LA aromatic protons: (a) on GA and (b) AA concentration.

The latter, i.e., the decrease in MA reactivity, was detected by the decrease in the CIDNP intensity of the aromatic protons. As compared to MA, lappaconitine, being the *N*-acetyl-anthranilic acid ester, is photolyzed via a more complex mechanism which, however, also includes the deacylation step.[34] Recently, the method of photo-CIDNP (including a time-resolved variant) has been applied to establish the detailed mechanism of LA phototransformation in homogeneous water−alcohol solutions.[34] Scheme 1 shows the radical intermediates and final products of lappaconitine phototransformation, *N*-acetyl-anthranilic acid (**II**) and enamine (**III**). The deacylation product (**IV**) is formed in minor amounts in homogeneous solutions and detected only in acidic media.

It was shown that LA phototransformation occurs from the triplet excited state I*.[34] The mechanism of formation of the main products II and III includes three steps: (1) intramolecular electron transfer from the nitrogen atom (N-20) to the anthranilic fragment to form a charged biradical followed by the charge exchange of this biradical with a lappaconitine molecule in the bulk resulting in a radical ion pair (RIP); (2) proton transfer from the 19-CH_2 group to the anthranilic fragment with formation of a neutral radical pair (V + VI); (3) fragmentation of neutral radicals resulting in formation of final products, the *N*-acetyl-anthranilic acid (*II)* and compound **III**.

In terms of the above-mentioned results of the influence of GA on MA photodecomposition,[18] it was assumed that the interaction between LA and GA leads to changes in the efficiency of the process of deacylation. It might be suggested

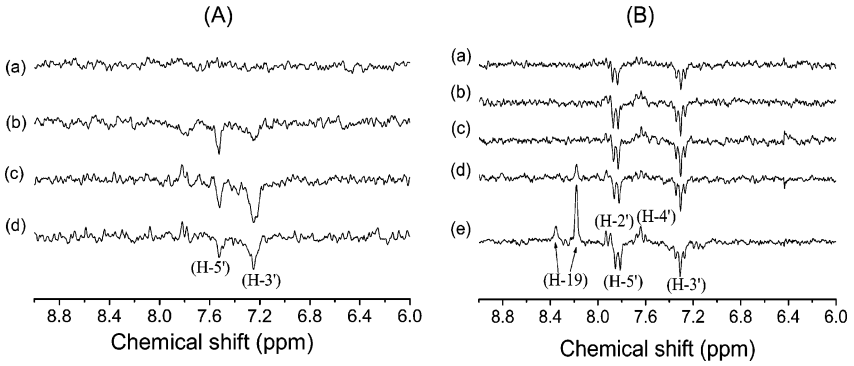


Figure 9. (A) Partial QSS CIDNP spectra detected during the photolysis of 1 mM LA at different GA concentrations in 20% CD_3OD: (a) 2, (b) 1, (c) 0.5, and (d) 0 mM. (B) Partial QSS CIDNP spectra of 0.5 mM LA at different GA concentrations in 50% CD_3OD: (a) 2, (b) 1, (c) 0.5, (d) 0.2, and (e) 0 mM.

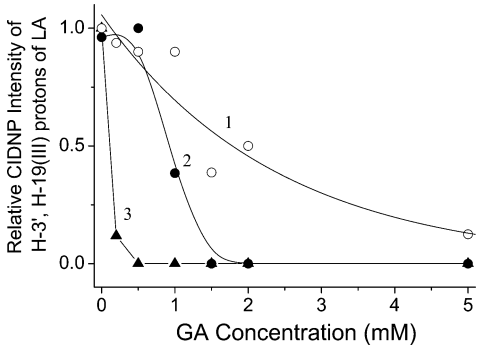


Figure 10. Dependence of the relative CIDNP intensity detected during the photolysis of 1 mM LA of (1) H-3′, (3) H-19 protons of **III** in 50% CD_3OD, (2) H-3′ in 20% CD_3OD on GA concentration.

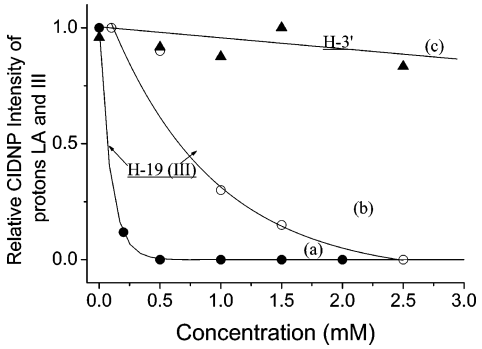


Figure 11. Dependence of the relative CIDNP intensity of the H-19 proton of **III** in 50% CD_3OD (a) on GA concentration and (b) on AA concentration and (c) of the H-3′ proton of LA in 50% CD_3OD on AA concentration.

that solubilization in the micelle should block processes involving the electron-transfer step, too.

We studied the GA effect on the photochemical activity of LA using the CIDNP method. In the previous study it was demonstrated that the deacylation reaction demonstrates polarization of aromatic protons of LA at the 3′ and 5′ positions (see Scheme 1).[34] Polarization, in the processes involving an electron-transfer step, was observed for the protons of compound **III** (H-19 and H-21). In this case CIDNP is generated in a radical ion pair (RIP, see Scheme 1) and neutral radical pair (**V** + **VI**). Thus, we assume that investigation of the dependencies of intensities of CIDNP of H-3′ aromatic protons and the H-19 proton on GA concentration in water−methanol mixtures allows one to test the influence of GA on separate stages of LA phototransformation.

Figure 8a shows the dependence of the CIDNP intensity of the H-3′ aromatic protons of the LA on GA concentration. Comparison of Figures 8a and 3A demonstrates the relationship between the polarization intensity of LA aromatic protons and the mole fraction of GA molecules in the micellar state. Thus, solubilization of LA in the micelle changes its nearest environment from the hydrophilic (in the homogeneous solution) to the hydrophobic one which in turn (as in the case of MA) leads to reduction of the intramolecular hydrogen bond which prevents deacylation. It might be the reason for the decrease in the polarization intensity with increasing GA concentration.

Taking into account that GA is a weak acid ($pK_{a1} = 4.4$, $pK_{a2} = 5.3$, $pK_{a1} = 6.9$)[35] it is necessary to verify that the observed effect of GA is not stimulated by the changes in the acidity of solution. To this end, the effect of GA was compared with those of acetic acid with a similar pK ($pK_a = 4.76$).[36] Figure 8b shows the dependence of the LA polarization intensity on the concentration of the added acetic acid. Comparing Figure 8a and b indicates that the observed change in polarization intensity on GA concentration cannot be assigned only to medium acidity, which additionally confirms the assumption of the effect of GA micelle formation on the reactivity of solubilized compounds.

Unfortunately, under the conditions when GA forms micelles, i.e., in 20% methanol solution, only the deacylation process is observed (CIDNP signals are recorded for H-3′ and H-5′ protons only) (Figure 9A). To study the influence of GA on the electron- and proton-transfer stages that lead to cleavage of the ester bond and formation of compounds **II** and **III**[34] we measured CIDNP intensities in the photolysis of LA in 50% methanol solution in the presence and absence of GA.

An increase in the fraction of methanol in a solution (Figure 9B) up to 50% leads to the manifestation of all steps of the above-mentioned mechanism. Therefore, the GA effect was studied for this solution. We measured the CIDNP intensity of two lines, H-19 (**III**) which reflects the efficiency of intramolecular electron transfer and H-3′ (**I**) which reflects the efficiency of deacylation.

Figure 10 shows the dependences of the relative CIDNP intensity of the H-3′ and H-19 protons on GA concentration in 50% water−methanol solution. The decrease in the efficiency of both processes, deacylation and electron transfer, was detected in the presence of GA (Figure 10(1,3)). Note that no micelle formation was detected under this experimental condition. As follows from Figure 10(3), an increase in GA concentration causes a sharp decrease in the CIDNP intensity of the H-19 proton of LA. A decrease is also observed for the H-3′ LA aromatic proton (Figure 10(1)) which is, however, smoother.

One might suggest that more drastic changes in the CIDNP of H-19 is due to a contribution from protonation of the nitrogen

atom N-20. This should decrease the efficiency of electron transfer. However the effect of GA on the CIDNP of H-19 is much stronger compared to the effect of acetic acid on this process (see Figure 11b). Thus, the influence of GA on the efficiency of the electron- and proton-transfer processes could not be explained by the action of GA acid function. In our opinion this points to the fact that the absence of micelles does not mean the absence of binding of GA to LA. Earlier, formation of LA−GA complexes with a 1:2 stoichiometry was detected at GA concentrations in the range 10^{-5}−10^{-3} M by optical methods.[19] Figure 11a,b additionally confirms the fact that GA does not play only the role of an acid for the electron-transfer processes.

4. Conclusion

Thus, NMR and CIDNP studies of LA photolysis in water−alcohol solutions with and without GA demonstrate high sensitivity of the phototransformation processes to the effect of medium in general and GA in particular. It is shown that in the 80% water−alcohol solutions solubilization of LA by glycyrrhizic acid suppresses one of the photolysis directions, namely, deacylation. It is also demonstrated that in the 50% water−alcohol solutions a specific interaction was detected between GA and LA that differs from LA solubilization in the GA micelle.

Taking into account that the main metabolite of the LA-based drug is the deacylated form of LA,[37] the blocking of the deacylation process by GA observed in the present paper might clarify the nature of the therapeutic action of the complex formation between the drug and GA. One might suggest that a decrease of the deacylation rate of the complexes of LA with GA is the one of the reasons for the decrease in the active dose of LA in the presence of GA.[37]

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