Molecular Interaction between Andrographolide and Glutathione Follows Second Order Kinetics

Zhiqiang ZHANG,[1)] Gallant Kar-Lun CHAN, Jieliang LI, Wang-Fun FONG, and Hon-Yeung CHEUNG*

Research Group for Bioactive Products, Department of Biology and Chemistry, City University of Hong Kong; Hong Kong SAR, China. Received August 17, 2007; accepted May 22, 2008; published online June 5, 2008

The intracellular level of glutathione (GSH) was significantly decreased after the addition of andrographolide (1) to cell cultures of HepG2. When the molecular interaction between andrographolide and GSH was investigated under a condition mimicking the *in vivo* environment, we observed that the level of GSH dropped in the presence of andrographolide. Stoichiometric analysis indicates that the reaction between these two reactants was 1 to 1 at pH 7 and followed second order kinetics. The activation energy of the overall reaction was $41.9 \pm 10\ \mathrm{kJ \cdot mol^{-1}}$ according to the Arrhenius equation. Using a micro-liquid–liquid extraction method followed by micellar electrokinetic chromatographic separation, two major products were isolated and identified, and their chemical structures were determined as 14-deoxy-12-(glutathione-amino)-andrographolide (2) and 14-deoxy-12-(glutathione-*S*-yl)-andrographolide (3). Based on these structural findings, a hypothetical mechanism of reaction between glutathione and andrographolide was proposed. It is concluded that the α,β-unsaturated lactone moiety of andrographolide reacts with GSH through a Michael addition followed by dehydration of the adduct.

Key words andrographolide; glutathione; Michael addition; reaction rate constant

Glutathione, a tripeptide biocompound containing a free thiol functional group, is abundant in cells. Because of the free thiol moiety, it plays a major protective role against oxidative stress. Glutathione exists in two forms. The antioxidant "reduced glutathione (GSH)" is conventionally called glutathione while the oxidized form is a sulfur–sulfur linked compound, known as glutathione disulfide (GSSG). GSH is an important intracellular reductant which is able to breakdown hydrogen peroxide (H_2O_2) through a non-enzymatic process. Depletion of GSH has been reported to trigger suicide of a cell by a process known as apoptosis.[2,3)] Hence, the intracellular level of GSH appears to be a sensitive indicator of the overall health status of a cell as it reflects the ability of the cell to resist toxic challenges.

Amongst all herbal substances widely used as functional foods or folk medicines in Asia, many of them are rich in polyphenols or phenolic acids and capable of cleansing the superoxide radicals formed in a cell. Consequently, they could be used as a chemoprophylaxis agent.[4)] However, a herbal plant called *Andrographis paniculate*, which is a rich source of andrographolide but not polyphenols or phenolic acids, has been long used as a folk medicine for alleviating inflammatory disorders,[5,6)] for hepatic protection[7—9)] and for antiproliferative purpose of cancer.[10—12)] Andrographolide has been considered as an inhibitor of nuclear factor (NF)-κB.[13)] Pharmacological research revealed that the andrographolide attenuates inflammation by inhibition of NF-κB through alkylation of reduced cysteine 62 of p50.[14)] Recent studies carried out by our group demonstrated that andrographolide induced the increase of reactive oxygen species (ROS) (mainly H_2O_2) level in HepG2 cells.[15)] This may be attributed to a reaction between andrographolide and GSH. The kinetics of the reaction between andrographolide and GSH, therefore, was investigated in order to obtain preliminary and basic information on the chemical modification of peptides and proteins with andrographolide. Our results reveal that andrographolide reacted with GSH to form a dehydrated adduct.

Results and Discussions

Kinetics of the Bimolecular Reaction between Andrographolide and GSH Simultaneous analysis of andrographolide and GSH is not easy due to the great differences in polarity of the two chemicals. After comparing two reported methods, namely micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE) which had been used for the detection of andrographolide and GSH, the former method was chosen for the investigation of the reaction because it was more sensitive. A calibration curve was constructed and the peak area of andrographolide in the MEKC chromatogram was plotted against the concentrations of standard solutions of andrographolide ranging from 0.1 to 0.5 mM. Within this concentration range, a good linear regression ($R=0.996$) was obtained. The good linear regression indicated that it was a reliable analytical method.

Before the kinetic study, the stoichiometry of the reaction between andrographolide and GSH was investigated using continuous variation method. Briefly, in 800 μl phosphate buffer (pH=7.0, 10 mM), $25n$ μl andrographolide (0.01 M) and $25(8-n)$ μl GSH (0.01 M) were added (where $n=0$, 1, 2, 3, 4, 5, 6, 7, 8), respectively. After being maintainted at 50 °C in water bath for 5 h, the series of mixtures were analyzed by CE. The amount of products in the mixtures was determined by the area of the peaks of products in the CE chromatogram. When the amount of products was plotted *versus* the molar fraction of andrographolide in the series of mixtures, the Job-plot (see Fig. 1) was obtained. As shown in Fig. 1, the maximal amount of products appeared at the 0.5 molar ratio of andrographolide. Therefore, the stoichiometry of the reaction was estimated as 1 molecule of andrographolide for 1 molecule of GSH.

The kinetics of reaction between andrographolide and GSH was determined at four different temperatures, *i.e.,*

* To whom correspondence should be addressed. e-mail: bhhonyun@cityu.edu.hk

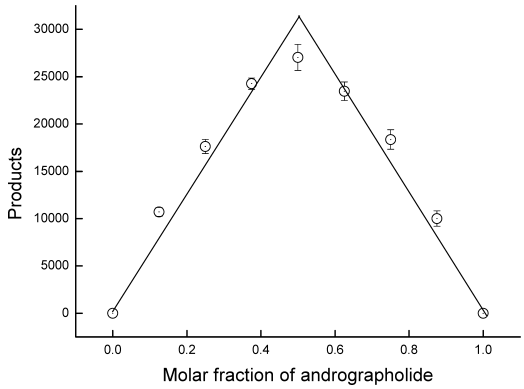

Fig. 1. Continuous Variation Plot of the Reaction between Andrographolide and GSH

The products were represented by the area (in arbitrary unit) of peaks of products in CE chromatogram. The molar fraction of andrographolide is calculated as $V_{andro}/(V_{andro}+V_{GSH})$, where the V_{andro} and V_{GSH} are the initial volumes of andrographolide solution and GSH solution in the series of mixtures.

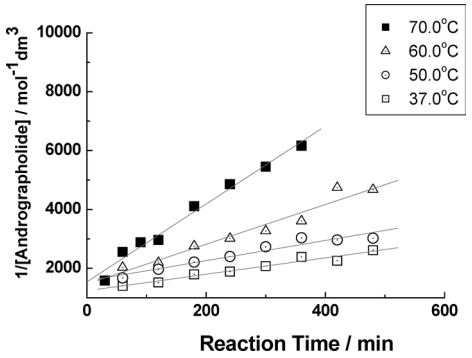

Fig. 2. Plots of the Reciprocal of Concentration of Andrographolide *versus* Reaction Time at 37, 50, 60, 70 °C

The degradation rate of andrographolide for each temperature can be expressed as $1/c=1240+2.79t$, $R=0.979$; $1/c=1580+3.42t$, $R=0.967$; $1/c=1470+6.75t$, $R=0.974$; $1/c=1540+13.2t$, $R=0.991$.

37.0, 50.0, 60.0 and 70.0 °C. Andrographolide and GSH were mixed well in phosphate buffer (20 mM, pH=7.0) and then incubated in a water bath at a set temperature. Initial concentrations of andrographolide and GSH were both 1 mM. The consumption of andrographolide during the observation period from 0.5 to 8 h fitted second order kinetics, in which the linearity was best met when the reciprocals of andrographolide concentration were plotted against the reaction time (Fig. 2). The reaction rate constants (K_T) for each temperature determined from the slope of the fitted lines were 2.8 ± 0.4, 3.4 ± 0.8, 6.7 ± 1.2 and 13.2 ± 1.4 mol$^{-1}\cdot$dm$^3\cdot$min^{-1}, respectively. Activation energy of the overall reaction was estimated as 41.9 ± 10 kJ$\cdot$mol^{-1} from the Arrhenius plot (Fig. 3) which is the relationship of $\ln K$ and the reciprocal value of the thermodynamic temperature (T).

Identification of the Reaction Products In order to identify the reaction products, 0.35 g andrographolide and 0.3 g GSH were dissolved in 20 ml ethanol and 10 ml phosphate buffer (0.2 M, pH=7.5), respectively. The two solutions were mixed and incubated overnight in a water bath at 50 °C and then after extraction three times with CH_2Cl_2, the mixtures were subjected to isolation using RP C-18 HPLC (mobile phase: methanol 20%, 0.05% v/v TFA solution 80%). Two major reaction products (compounds **2** and **3** in Fig. 4),

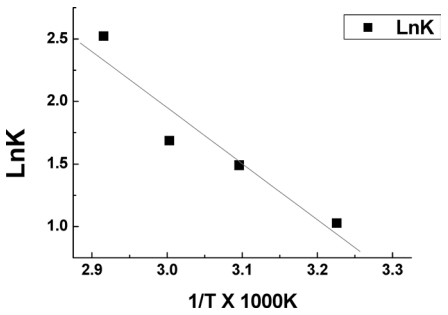

Fig. 3. Arrhenius Plots for the Heat Activation between Andrographolide and GSH

The Arrhenius plot is expressed as $\ln K=17-5.05\times10^3\,T^{-1}$, $R=-0.951$.

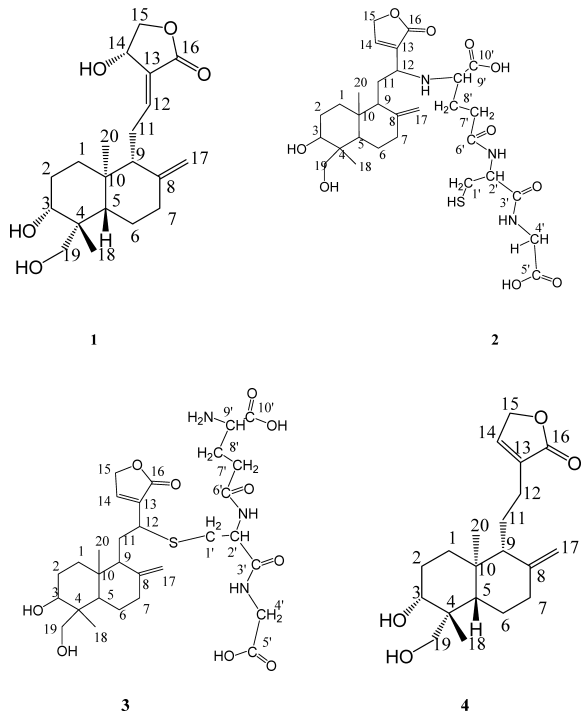

Fig. 4. Chemical Structures of Compounds **1**—**4**

Compound andrographolide (**1**), 14-deoxy-12-(glutathione-amino)-andrographolide (**2**), 14-deoxy-12-(glutathione-*S*-yl)-andrographolide (**3**), 14-deoxy-andrographolide (**4**).

were purified. The structure of these two products was established by spectroscopic methods. Both compounds **2** and **3** were less polar than GSH as they had longer retention times on HPLC. On the other hand, in contrast to andrographolide which is very hydrophobic, these two products were water soluble.

Compound **2**, which occurs as white amorphous powder after freeze dried, was positive to the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone. The FT-Raman spectrum indicates the presence of hydroxyl (2940 cm^{-1}) and ester carbonyl (1644, 1446 cm^{-1}) groups in the molecule. Two major fragment peaks were observed at $m/z=638.5$ and $m/z=306.2$ in the negative ESI-MS spectrum. These two peaks were assigned as ion $(M-H)^-$ and $(GSH-H)^-$, respectively. According to the ESI-MS spectrum, the molecular formula of compound **2** was $C_{30}H_{45}O_{10}N_3S$ and the calculated molecular weight of **2** is 639.46, which was 18 mass (H_2O) units smaller than the molecular weight

addition of andrographolide and GSH. Compound **2**, therefore, might be a dehydrated adduct of andrographolide and GSH. This conclusion received further supports from data of the ^{1}H-NMR (300 MHz; D_2O) spectra. Comparing the NMR of this compound to that of andrographolide (300 MHz; DMSO-d_6), a very special signal which was δ= 7.43 (s, H-14) was observed at downfield. On the other hand, a doublet signal at δ 6.83, originally assigned to H-12 of andrographolide, disappeared in the spectrum of the former. These changes in chemical shift indicated that the original olefin bond at C-12 and C-13 in andrographolide moiety had shifted to C-13 and C-14 in compound **2** and the C-12 of andrographolide moiety covalently connected with GSH. The GSH molecule possesses two potential active sites—the amino group of glutamic acid peptide and the thiol of cysteine peptide. The chemical shift of the proton at C-12 of andrographolide moiety was 3.54 ppm, which suggested the C-12 was more likely connected with an amine group. Based on this information, compound **2** was determined to be 14-deoxy-12-(glutathione-amino)-andrographolide.

Compound **3**, which was also a white amorphous powder, possesses an α,β-unsaturated lactone because it was positive to the Legal and Kedde reactions. The FT-Raman spectrum of compound **3** is very similar to that of **2** but had peaks at 2940, 1644 and 1446 cm^{-1}. When the negative ESI-MS was applied to detect the molecular weight of compound **3**, it gave two fragment ions at m/z=638.5 and m/z=306.2 which were the same as that of compound **2**. Therefore compounds **3** and **2** might be isomeric and this was confirmed when the ^{1}H-NMR (300 MHz, D_2O) and g-COSY data of compound **3** were taken into account. The ^{1}H-NMR data of compounds **3** and **2** shows close similarity except for H-12 and some other slight differences. The proton signal of H-12 in compound **3** shifted toward up field about 50 Hz compared to that of compound **2**, suggesting that it was a sulfur but not a nitrogen atom that combined the GSH moiety to C-12 of andrographolide in compound **3**. In the g-COSY spectrum, a stepwise coupling from H-9 (δ 1.76, 1H, overlapped) through H-12 (δ 3.35, 1H, m) mediated by H-11 (δ 1.68, 2H, overlapped) and an independent vicinal coupling from H-14 (δ 7.42, 1H, s) and H-15 (δ 4.74, 2H, s) was observed. The ^{1}H-NMR spectrum of compound **3** was similar to 14-deoxy-12-(cysteine-*S*-yl)-andrographolide-3-*O*-sulfate,[16] which is one of the metabolites of andrographolide separated from human urine. According to the above analyses, the compound **3**, an isomer of **2**, was determined to be 14-deoxy-12-(glutathione-*S*-yl)-andrographolide, as shown in Fig. 4.

Reaction Mechanism Here, we propose that the formation of dehydrated adducts, compounds **2** and **3**, possibly resulted from two-stepped reactions which begin with the Michael addition at C-12 of andrographolide to form an anion intermediate. Subsequently, the final reaction products were formed when the allylic hydroxyl was eliminated from C-14. The reaction scheme proposed is illustrated in Fig. 5. According to this scheme, the glutamic acid amino (:NH_2) of GSH attacks the electrophilic C-12 of andrographolide leading to the formation of compound **2**. On the other hand, the C-12 could similarly be attacked by cysteine thiolate ion (S^-) of GSH to form an intermediate which is the precursor of compound **3**. Since the nucleophiles, amino and thiolate ion, only present stably in alkali environments, the andrographolide and GSH would tend to react in alkali solvent. We analyzed the reaction of andrographolide with GSH in four different phosphate buffers, pH=6.5, 7.0, 7.5, and 8.0. The reaction rate and the ratio of **3** to **2** were elevated with the increase of pH value that could support the reaction mechanism proposed above.

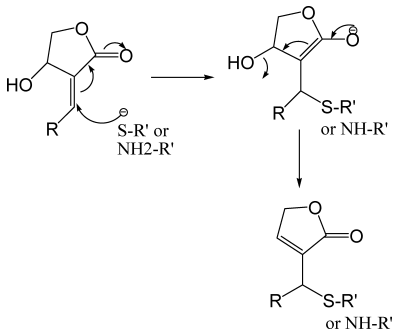

Fig. 5. Proposed Mechanism of the Reaction between Andrographolide and GSH

The nucleophilic addition, in frontier orbital terms, involves an interaction between the HOMO of nucleophile and LUMO of electrophile. The molecular orbitals and atom charges of andrographolide were analyzed from the optimized geometry, which was modeled at the HF/6-31G (D) theory level. The natural population analysis (NPA) charges of C-12, C-13, and C-16 are all positive, which suggested these atoms are electrophilic, and hence, can be attacked by a nucleophile. In this case, it was glutamic acid NH_2 group or cysteine thiolate ion. All calculations were carried out using the Gaussian software package.[17] The LUMO of andrographolide were composed mostly by P-orbital of C-12, C-13, C-16 and the carbonyl oxygen atom, and the biggest participant was the C-12 at which the nucleophilic addition would most possibly take place. Further more, the phase of the electron cloud of C-12 and oxygen was opposite to C-13 and C-16. That is to say, the P-orbitals of C-13 and C-16 stand side by side to form a unit which would exclude the two opposite P-orbital from C-12 and oxygen atom. When the C-12 was attacked by a nucleophile, the LUMO of andrographolide would be filled by electrons from the nucleophile's HOMO resulting in a transfer of the C–C double bond from C-12, C-13 to C-13, C-16. The electron migration direction, which is the first step of the reaction, is shown in Fig. 5. In the second step, the hydroxyl at C-14 would leave the intermediate generated in the first step, and the C–C double bond between C-13, C-16 would relocate to C-13, C-14.

As discussed above, the α,β-unsaturated lactone could be attacked by nucleophiles especially the free sulfhydryl and amine group. This profile gives these compounds cytotoxicity.[18,19] The structural cytotoxicity relationship of andrographolide has been studied by Nanduri *et al.*[20] They proposed that the moieties: α-alkylidene γ-lactone ring, allylic hydroxyl at C-14, olefin bond at C-12, C-13, and double bound or epoxy at C-8, C-17 are responsible for the cytotoxic activity exhibited by andrographolide and its analogs. In our experiments, 14-deoxy-andrographolide, another bioactive component of *Andrographis paniculate*, was investigated as well. However, it hardly reacts with GSH under the same conditions as andrographolide. The structures of andro-

grapholide and 14-dexoy-andrographolide are very similar except that the former possesses an exocyclic C=C bond while the latter possesses an incyclic C=C bond in their α,β-unsaturated lactone moiety.

***In Vitro* Interaction between Andrographolide and GSH** Some intracellular proteins regulate signal transduction by glutathionylation. Whenever the redox stage of a cell changed, formation of mixed disulfides between proteins' cysteine and glutathione occurs. The reduced glutathione thus, either plays as an antioxidant and a free radical scavenger to protect the cell or to affect signal transduction at gene expression level so that apoptosis of the cell could be prevented. In addition, GSH is an important antioxidant especially in the metabolism of H_2O_2. The sulfhydryl group of the cysteine residue in GSH can reduce the H_2O_2 to water and oxidized form of sulfur. Andrographolide could induce the accumulation of H_2O_2 *in vitro* indicating that it may interact with the action of GSH.[15] This kinetic study demonstrated that andrographolide could react with GSH, and the reaction included two branches. The first reaction branch led to the generation of compound **2**, and the second one, which might be responsible for the bioactivity of andrographolide, resulted in the formation of compound **3** which is an alkylate to the reduced sulfhydryl of GSH. The *in vitro* interaction between andrographolide and GSH were studied in this work. The result indicates that andrographolide could cause the depletion of the intracellular GSH level (shown in Fig. 6) in cell culture of HepG2 which is a type of human liver cancer cell. The level of GSH in 100 μM andrographolide-treated cells after 12 h was only half of the control level.

It seems contradictory between the hepatoprotective activity of andrographolide and the effect on depleting intracellular GSH. The hepatoprotective activity of andrographolide is related to its antioxidant activity. Nevertheless, the interaction between andrographolide and GSH will reduce the antioxidant activity. This indicates that andrographolide may have dual redox activity. It has been reported that the α,β-unsaturated lactone could scavenge the superoxide anion (O_2^-).[21] Therefore, andrographolide can act as an antioxidant reducing the oxidative free radical. On the other hand, andrographolide reacts with intracellular GSH resulting in the increase of oxidative stress in cells. This could explain why the hepatoprotection of andrographolide is less effective than its analogue, *i.e.,* neoandrographolide (a glucoside of 14-deoxyandrographolide).[22] Because the incyclic C=C bond is more inert than the exocyclic one, neoandrographolide is unable to cause the significant depletion of GSH as andrographolide does. Consequently, it gives neoandrographolide a better hepatoprotective ability than andrographolide.

Conclusion

The kinetics of the reaction between andrographolide and GSH was investigated using the MEKC method. Bimolecular reaction rate constants were obtained at 37.0, 50.0, 60.0 and 70.0 °C. The reaction rate constants were 2.8±0.4, 3.4±0.8, 6.7±1.2 and 13.2±1.4 $mol^{-1}\cdot dm^3\cdot min^{-1}$, respectively. According to the Arrhenius law, the activation energy of the reaction between andrographolide and GSH was determined to be 41.9±10 $kJ\cdot mol^{-1}$. Two major products, *i.e.* compounds **2** and **3**, were separated and purified by reverse phase HPLC. Their structures were established by spectral methods. The reaction mechanism was proposed based on the population analysis to andrographolide using computational modeling. The formation of the addition products may undergo a two-stepped reaction: nucleophilic adduction at C-12 of andrographolide followed by the leaving of a hydroxyl group from C-14 of andrographolide.

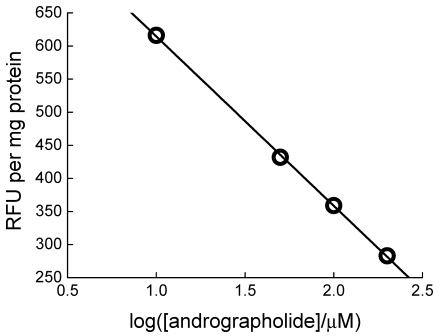

Fig. 6. Effect of Andrographolide on the Intracellular GSH Level of HepG2 Cells

Cells were treated with indicated concentrations of drug for 12 h and then the GSH level was determined using monochlorobimane as a fluorescence indicator which binds specifically to GSH. The fluorescence was measured at 380/460 nm. RFU: random fluorescence unit.

Both reaction rate constant and activation energy are two key physical chemical data which indicate how fast and how difficult a chemical reaction may take place. The former is related to the rate of a reaction while the later reflects the threshold of a chemical reaction. Information of these two aspects can help us understanding the metabolism and the bioavailability of a drug in a biological system. Although andrographolide described in this study is an alkylating agent, which could covalently bind to biological macromolecule, cellular thios moiety could also substantially influence the availability of a drug for binding to macromolecules. GSH is an important intracellular thio-compound possessing antioxidating property in the cells. The values obtained in this study, therefore, are fundamental for exploration of the bioactivity of andrographolide. It allows us to assess the *in vivo* redox status in the presence of andrographolide.

Experimental

Chemicals and Reagents All chemicals used in this experiment were commercial products from several companies. Purified andrographolide (97 %) was purchased from Indofine (U.S.A.). The reduced form of L-glutathione (>99%) and sodium phosphate monobasic monohydrate were obtained from Sigma-Aldrich (Germany), while sodium phosphate dibasic heptahydrate and sodium dodecyl sulfate (SDS) were purchased from US Biochemicals Ltd. (U.S.A.). All solvents used, including methanol and ethanol, were of analytical grade. They were purchased from Riedel-de Haën (Germany).

Andrographolide was dissolved into ethanol at concentration of 0.01 M and stored at 4 °C for further use. The GSH standard solution was prepared by dissolving the chemical in water at the same concentration as andrographolide. In experiments, the standard solution was diluted to the proper concentration.

Micellar Electrokinetic Chromatography (MEKC) A capillary electrophoretic (CE) apparatus (Model: P/ACE MDQ), from Beckman Coultler (Fullerton, CA, U.S.A.), was used for monitoring the concentration of andrographolide. The CE was equipped with a 50 μm I.D.×57 cm (50 cm from inlet to detector) fused-silica capillary tube. Instrumental setting and data analysis were controlled by the MDQ 32Karat software developed by Beckman. The CE system was operated using an electrical voltage at 20 kV, and capillary temperature at 25 °C, while 214 nm was selected as detection UV–visible wavelength. Samples were injected under a pressure of 0.5 psi, for

5 s. The buffers[23,24] used in CE separation were composed of 15 mM SDS, 20 mM borate buffer (pH=9.5) and 5% (v/v) methanol.

Reaction Products Separation and Identification To isolate the reaction products, 0.35 g andrographolide and 0.3 g GSH were dissolved in 20 ml ethanol and 10 ml phosphate buffer (pH=7.5, 0.2 M), respectively. The two solutions were mixed and incubated overnight in a water bath at 50 °C. After three times of extraction by CH_2Cl_2, the mixture was subjected to isolation using RP C-18 HPLC (mobile phase: methanol 20%, 0.05% v/v TFA solution 80%). Flow rate of the mobile phase was set as 1 ml/min and the detection wavelength was 210 nm. The FT-Raman spectrum was obtained at the PERKIN ELMER System 2000 NIR FT-Raman. The mass spectrum was performed with the Electrospray Ionisation Mass Spactrometry (ESI-MS) at PE SCIEX API 365 system.

Compound 2 Amorphous substance. ^{1}H-NMR (300 MHz, D_2O) δ: 0.42 (3H, s; H-20), 0.81 (1H, m; H-1), 0.89 (3H, s; H-18), 0.96 (1H, d, J=13.2 Hz; H-5), 1.09 (1H, m; H-1), 1.23 (1H, d, J=11.4 Hz; H-6), 1.52 (2H, overlapped; H-2), 1.56 (2H, overlapped; H-11), 1.63 (1H, d, J=13.2 Hz; H-6), 1.69 (1H, m, o; H-9), 1.88 (1H, d, o, J=13.8 Hz; H-7), 1.89 (2H, m, o; H-8′), 2.17 (1H, d, J=13.8 Hz; H-7), 2.28 (2H, m; H-7′), 2.55 (1H, dd, J=14.4, 8.4 Hz; H-1′), 2.82 (1H, dd, J=14.4, 4.8 Hz; H-1′), 3.16 (1H, m, o; H-3), 3.20 (1H, d, J=11.4 Hz; H-19), 3.49 (1H, overlapped; H-9′), 3.51 (2H, overlapped; H-4′), 3.54 (1H, overlapped; H-12), 3.80 (1H, d, J=11.4 Hz; H-19), 4.28 (1H, dd, J=8.1, 4.8 Hz; H-2′), 4.40 (1H, s; H-17), 4.72 (1H, s; H-17), 4.75 (2H, s; H-15), 7.43 (1H, s; H-14). Negative ESI-MS m/z 638.5 $[M-H]^-$ (Calcd for $C_{30}H_{45}O_{10}N_3S$ 639.46).

Compound 3 Amorphous substance. ^{1}H-NMR (300 MHz, D_2O) δ: 0.42 (3H, s; H-20), 0.93 (3H, s; H-18), 1.05 (2H, overlapped; H-1), 1.08 (1H, overlapped; H-5), 1.60 (4H, m, overlapped; H-6, H-2), 1.68 (2H, overlapped; H-11), 1.76 (1H, overlapped, H-9), 1.82 (1H, m; H-7), 1.93 (2H, m; H-8′), 2.20 (1H, d, J=12.6 Hz; H-7), 2.30 (2H, m; H-7′), 2.50 (1H, m; H-1′), 2.71 (1H, m; H-1′), 3.22 (1H, d, J=11.7 Hz; H-19), 3.26 (1H, m, overlapped; H-3), 3.35 (1H, m; H-12), 3.51 (2H, m, overlapped; H-4′); 3.53 (1H, overlapped; H-9′), 3.82 (1H, d, J=11.7 Hz; H-19), 4.16 (1H, m; H-2′) 4.29 (1H, s; H-17), 4.67 (1H, s; H-17), 4.74 (2H, s; H-15), 7.42 (1H, s; H-14). Negative ESI-MS m/z 638.5 $[M-H]^-$ (Calcd for $C_{30}H_{45}O_{10}N_3S$ 639.46).

Measurement of the Intracellular Level of GSH The intracellular GSH levels in cell culture of HepG2 were measured using a Glutathione Detection Kit (Chemicon, Temecula, CA, U.S.A.) with the mothod described previously.[14]

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