ISSN 0003-6838, Applied Biochemistry and Microbiology, 2010, Vol. 46, No. 1, pp. 23–27. © Pleiades Publishing, Inc., 2010.
Original Russian Text © A.G. Sysa, P.A. Kiselev, V.N. Zhabinskii, V.A. Khripach, 2010, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2010, Vol. 46, No. 1, pp. 29–34.

Effect of the Structure of the Brassinosteroid Side Chain on Monooxygenase Activity of Liver Microsomes

A. G. Sysa, P. A. Kiselev, V. N. Zhabinskii, and V. A. Khripach

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Minsk, 220141 Belarus;
e-mail:Aliaksei.Sysa@gmail.com

Received December 4, 2008

Abstract—Possible pathways by which brassinosteroids affect the monooxygenase enzymatic system of mammalian liver microsomes, which is involved in the transformation of a broad spectrum of xenobiotics, were studied. The role of the structure of the side chain of brassinosteroids in the regulation of monooxygenase activity was studied using two natural compounds (24-epibrassinolide and 28-homobrassinolide) and two synthetic analogues, (22S,23S-dihydroxy) stereoisomers. The results of this study show that brassinosteroids can directly influence the functioning of the microsomal enzymatic system. It was found that the degree of this influence depends on the side chain structure. This suggests the possibility of targeted modification of natural compounds to ensure the desired physiological effects.

DOI: 10.1134/S0003683810010035

INTRODUCTION

In recent years, the interest in brassinosteroids has significantly increased, because these phytohormones not only exhibit growth-stimulating activity but also increase the quality of plant products, reducing the accumulation of nitrates, heavy metals, and radionuclides in them and making it possible to decrease the environmental load compared to other conventional methods of plant protection [1–3]. Today, this group of steroids numbers over 70 compounds [3–5]. It should be noted that plant steroid hormones are similar to animal steroid hormones both in structure and functions: they regulate gene expression in plants and modulate metabolic processes, as well as cell growth and differentiation [6, 7].

However, since the studies on the effect of brassinosteroids on enzymatic reactions in animals are only at the initial stage, no substantiated inferences can be made regarding the molecular mechanisms of the potential effect of these compounds and the structural–functional relationship in enzymatic systems.

The purpose of this study was to determine the pathways by which brassinosteroids may influence mammalian enzymatic systems using two natural compounds, 28-homobrassinolide and 24-epibrassinolide, and their synthetic (22S,23S-dihydroxy) stereoisomers as an example. Another goal of this study was to determine the role of the side chain of brassinosteroids in their effect on the catalytic properties of the monooxygenase enzymatic system of liver cells, which is involved in the conversion of various endogenous and exogenous organic compounds in humans and animals, including the metabolic activation of polycyclic aromatic hydrocarbons into carcinogenic forms.

MATERIALS AND METHODS

Reagents. Tris-(oxymethyl)-aminomethane, glycerol, glycine, dimethyl sulfoxide, and trichloroacetic acid (TCA) were purchased from Reakhim (Russia). Fatty acid-free bovine serum albumin, dithiothreitol (DTT), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Serva (Germany). NaOH and KOH were from Lachema (The Czech Republic); 7-ethoxyresorufin was from Sigma (United States). 28-Homobrassinolide, (22S,23S-dihydroxy)-28-homobrassinolide, 24-epibrassinolide, and (22S,23S-dihydroxy)-24-epibrassinolide were obtained as described in [8, 9]. All compounds were characterized by IR spectra recorded with a UR-20 spectrophotometer (Carl Zeiss, Germany) in KBr pellets and films as well as by NMR spectra recorded with an Avance-500 spectrometer (Bruker, Germany) (500 MHz for ^{1}H nuclei and 125 MHz for ^{13}C nuclei) in $CDCl_3$ solutions in 5 mm standard ampoules. Mass-spectrometric characteristics of steroids were determined with a Varian MAT-311 mass spectrometer (Varian, United States) at an ionizing radiation energy of 70 eV.

Microsomal fraction isolated from the liver of three-month-old male rats weighing 200 g was used as a biochemical model. The animals were intraperitoneally injected with 20-methylcholanthrene (40 mg/day per kg body weight in kernel oil) in the first two days of the experiment and euthanized on the fourth day after the beginning of injections [10].

Isolation and characteristics of the rat liver microsomal fraction. The microsomal fraction was isolated as described in [11] with an additional centrifugation at 12000 *g* for 15 min at 4°C before subsequent

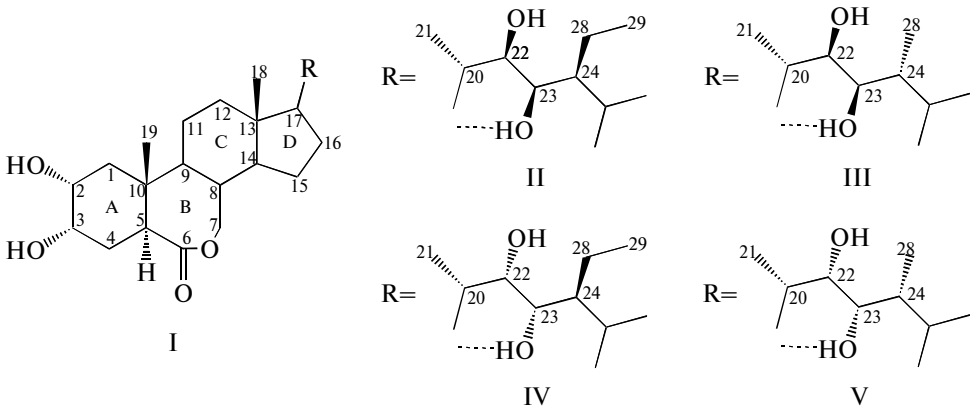

Fig. 1. Structure of brassinosteroids used in the study: (I) structure of brassinosteroid rings, (II) 28-homobrassinolide, (III) 24-epibrassinolide, (IV) (22S,23S)-28-homobrassinolide, and (V) (22S,23S)-24-epibrassinolide.

centrifugation at 100000 *g*. The pellet was resuspended in a buffer containing 0.01 M Tris-acetate (pH 7.4), 20% glycerol, 1 mM EDTA, and 1 mM DTT. The suspended pellet was frozen in liquid nitrogen and stored at –18°C. The content of cytochrome P-450 was determined by the standard method after the addition of $Na_2S_2O_4$ and bubbling with CO [12]. The protein concentration was measured using a kit from Piersce (Rockford, IL, United States). Thus obtained preparation usually contained 1.7 nmol cytochrome P-450 per milligram of microsomal protein.

Determination of the catalytic activity of cytochrome P-450 with respect to 7-ethoxyresorufin. Oxidative deethylation of 7-ethoxyresorufin was performed as described in [13] at 37°C in 0.05 M buffer (1 ml) containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and the rat liver microsomal fraction. The concentration of cytochrome P-450 was 27.5 pmol/ml. The initial concentration of the substrate in the majority of experiments was 0.5 μM. The reaction was started by the addition of NADPH. After 10 min of incubation, the reaction was stopped by the addition of 1 ml of acetone chilled to 4°C. The concentration of the reaction product (resorufin) was determined spectrofluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Determination of the catalytic activity of cytochrome P-450 with respect to benzo(a)pyrene. The activity of cytochrome P-450 was determined as described in [14] with some modifications. Briefly, benzo(a)pyrene was oxidized at 37°C in a buffer (1 ml) containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and microsomes of animals injected with 20-methylcholanthrene. The concentration of cytochrome P-450 was 55 pmol/ml. The reaction was started by the addition of NADPH and performed for 20 min. The initial concentration of benzo(a)pyrene was 1.0 μM. The reaction was stopped by the addition of 1 ml of cold acetone (0–4°C). To extract benzo(a)pyrene, 3.25 ml of hexane was added and the mixture was shaken for 1 min using a Œicro-Shaker-326m (Poland). An aliquot (1 ml) of the organic phase was taken and mixed with 1 N NaOH (2 ml). The concentration of the reaction product, 3-hydroxybenzo(a)pyrene, in the alkaline phase was determined spectrophotometrically at an excitation wavelength of 396 nm and an emission wavelength of 533 nm.

Obtaining the complexes of brassinosteroids with albumin. To obtain the complexes, an aliquot of 50 μM steroid solutions in dimethyl sulfoxide was added to 2% bovine serum albumin at a polyoxysteroid–protein molar ratio of 3 : 1.

RESULTS AND DISCUSSION

The structure of studied steroid compounds is shown in Fig. 1. It can be seen that the compounds had the same structure of all four rings but differed in the structure and configuration of the side chain. For example, the natural brassinosteroid 24-epibrassinolide contains the 24R methyl group, whereas 28-homobrassinolide contains the 24S ethyl group at this position. A characteristic feature of synthetic derivatives of both compounds was the S-configuration of carbon atoms at positions C22 and C23 of the side chain, which carry OH groups.

Note that a comparative analysis of the effect of organic compounds on biological systems in vitro is often hampered by their poor solubility in water and, as a result, by low and sometimes significantly differing bioavailability of tested compounds. To minimize this effect, in this study polyoxysteroids were used in complexes with albumin. The formation of complexes was characterized by intrinsic fluorescence spectra of the protein, on the basis of which the following parameters were calculated: $(I_{320}/I_{360})_{296}$; $B = (I_{320}/I_{360})_{280}$, and $\Delta = B - A$, where I_{320} and I_{360} are fluorescence intensities at the wavelengths 320 and 360 nm, respec-

Characteristics of intrinsic fluorescence spectra of bovine serum albumin and its complexes with brassinosteroids (the protein–steroid molar ratio, 1 : 3)

Object	$I_{320/296}$	$I_{360/296}$	$I_{320/280}$	$I_{360/280}$	A	B	Δ	$\lambda_{max/296}$, nm	$\lambda_{max/280}$, nm
Albumin	363	482	661	779	0.75	0.85	0.1	343	343
Albumin + 28-homobrassinolide	331	439	592	695	0.73	0.82	0.09	344	344
Albumin + (22S,23S)-28-homobrassinolide	373	511	653	806	0.73	0.81	0.08	344	344
Albumin + 24-epibrassinolide	344	464	625	760	0.74	0.82	0.08	344	344
Albumin + (22S,23S)-24-epibrassinolide	394	545	648	822	0.72	0.79	0.07	344	344

tively [15]. The values beyond parentheses indicate the fluorescence excitation wavelength.

According to [15, 16], parameters A and Δcharacterize the contribution of tryptophan and tyrosine residues, respectively, to the total fluorescence intensity of the protein. The obtained values of parameters A, B, and Δ and the position of fluorescence emission maxima at excitation wavelengths of 280 and 296 nm are summarized in the table.

On the basis of data presented in the table, it can be postulated that the addition to albumin of all studied brassinosteroids at a steroid–protein molar ratio of 3 : 1 was accompanied by significant changes in the protein fluorescence parameters ($p < 0.05$), which can be interpreted as conformational changes determined by the formation of the albumin–steroid complex.

At the same time, the similar values of parameters A, B, and Δ for the complexes of albumin with all studied compounds testify to the identity of the protein–steroid structures formed ($p > 0.05$).

Assessment of the effect of brassinosteroids on the oxidation of 7-ethoxyresorufin and benzo(a)pyrene by liver microsomes of animals induced with 20-methylcholanthrene. It is known that injection of animals with 20-methylcholanthrene results in enhanced synthesis and, hence, enrichment of the liver microsomal fraction with cytochrome P-450 isozymes involved in detoxication and bioactivation of procarcinogenic endogenous and exogenous compounds [10]. Detoxication and bioactivation have been substantiated most illustratively for benzo(a)pyrene [14, 17]. For example, benzo(a)pyrene oxidation by cytochrome P-450 with the formation of phenolic and diol derivatives, which, in turn, are substrates for transferases, is one of the main pathways by which procarcinogenic polycyclic aromatic compounds are eliminated from the body (i.e., detoxicated).

Conversely, the epoxidation of benzo(a)pyrene dihydroxy derivatives (e.g., 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene) yields compounds exhibiting strong carcinogenic properties, i.e., leads to carcinogenic bioactivation. 7-Ethoxyresorufin is a widely used substrate reliably reflecting the bioactivating function of cytochrome P-450 [18, 19]. It is logical to characterize the detoxicating efficiency of the monooxygenase system by the reaction of benzo(a)pyrene hydroxylation. Both these reactions were used in this study as test ones.

The quantitative parameter reflecting the strength of the effect of phytohormones on the enzymatic activity is IC_{50} (i.e., the inhibitor concentration that causes a twofold decrease in the reaction rate). In cases when the effect of the studied compound was weak, we determined the reaction rate in percent relative to the control at a concentration of the given compound of 250 μM.

In such studies, the substrate concentration equal or close to the Michaelis constant (K_M) is generally used. This parameter, determined in special experiments, was found to be 0.5 μM for 7-ethoxyresorufin and 0.7 μM for benzo(a)pyrene.

The dependences characterizing the strength of the effect of brassinosteroids on the reaction of 7-ethoxyresorufin dealkylation catalyzed by the monooxygenase system are shown in Fig. 2.

As can be seen from Fig. 2, the inhibitory effect on the ethoxyresorufin-O-dealkylating activity of the liver microsomal fraction was largely determined by the structure of the side chain of the compounds studied. For instance, the inhibitory effect of 24-epibrassinolide in the concentration range studied was almost absent. The rate of oxidative dealkylation of 7-ethoxyresorufin decreased by 55–60% in the presence of (22S,23S-dihydroxy)-24-epibrassinolide and 28-homobrassinolide at concentrations 200–250 μM. However, the change in the configuration of hydroxy groups in the natural 28-homobrassinolide at positions C22 and C23 from R to S dramatically enhanced the inhibitory effect. The IC_{50} values calculated for this compound was 25 μM.

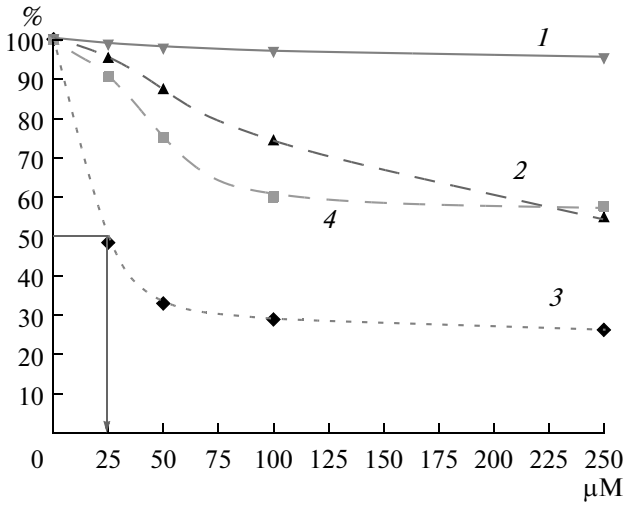

Fig. 2. Dependence of the rate of 7-ethoxyresorufin oxidation (% of control) by the monooxygenase system on the concentration (μM) of brassinosteroids: (*1*) 24-epibrassinolide, (*2*) 28-homobrassinolide, (*3*) (22S,23S)-28-homobrassinolide, and (*4*) (22S,23S)-24-epibrassinolide. The initial concentration of 7-ethoxyresorufin was 0.5 μM; the content of cytochrome P-450 was 27.5 pmol/ml. The reaction was performed in 50 mM Tris-HCl buffer (pH 7.4).

It should be noted that all the compounds studied had no significant effect on the detoxication of the procarcinogenic compound benzo(a)pyrene (Fig. 3).

Indeed, the inhibitory effect of epibrassinolide and homobrassinolide in the concentration range studied was not observed at all, and the addition of 250 μM of (22S,23S)-28-homobrassinolide and (22S,23S)-24-epibrassinolide caused only 35% inhibition.

Despite the relatively short history of studies of brassinosteroids, their exceptional role in the plant world causes no doubts. Many molecular mechanisms of action of these compounds have been established, which showed that brassinosteroids are the rate-limiting factor in cell development and that their functions are similar to the functions of human and animal steroid hormones [3–7]. The presence in the structures of brassinosteroids of α,α-hydroxy groups in ring A and the RR-configuration of the diol in the side chain is believed to be of principal importance: according to [20, 21], this structure ensures approximately 35 and 25%, respectively, contribution to the total activity of the relevant compound.

Much less is known on the role of ring B. It was shown that the activity of the steroid changed upon with the transition from the 7-oxalactone to the 6-keto cycle and completely disappeared in the case of the 6-oxalactone structure [22, 23]. The structural requirements to the type and configuration of substituent at position C24 are less principal [20]. All these requirements are associated with the structure of the active sites of receptors, through which hormonal reception in plants may take place. In this study, we attempted to assess the possibility of a direct effect of brassinosteroids on the monooxygenase enzymatic system of liver microsomes, which is involved in the transformation of a broad spectrum of xenobiotics, including the procarcinogenic polycyclic aromatic compounds of the benzo(a)pyrene type. To raise the content of cytochrome P-450 isozymes in the microsomal fraction (CyP1A1, CyP1A2, and CyP1B1), which exhibit an increased activity with respect to polycyclic aromatic hydrocarbons and can convert them into potent carcinogens, the animals were preliminarily injected with 20-methylcholanthrene, an inducer of all listed forms of cytochrome P-450 [25, 26].

To characterize monooxygenase activity, we used two substrates—7-ethoxyresorufin and benzo(a)pyrene. It was found that all the 20-methylcholanthrene-inducible isoforms of cytochrome P-450 exhibited a high catalytic activity with respect to the first substrate. The second one

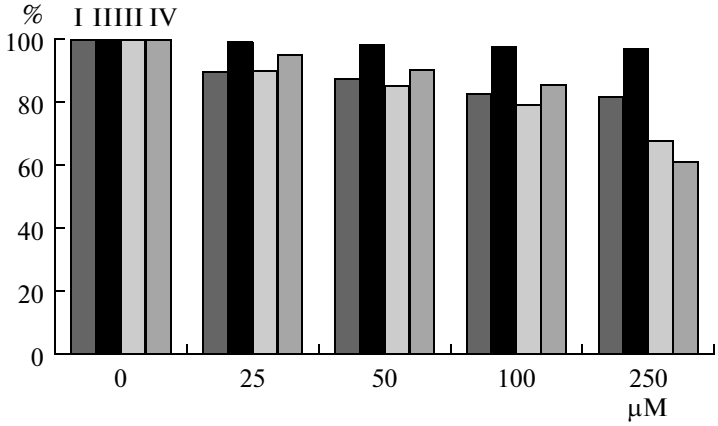

Fig. 3. Effect of brassinosteroids (μM) on benzo(a)pyrene oxidation (% of control): (I) 24-epibrassinolide, (II) 28-homobrassinolide, (III) (22S,23S)-28-homobrassinolide, and (IV) (22S,23S)-24-epibrassinolide. The initial concentration of benzo(a)pyrene was 0.5 μM; the content of cytochrome P-450 was 55.0 pmol/ml. The reaction was performed in 50 mM Tris-HCl buffer (pH 7.4).

was used to assess the detoxicating function of the monooxygenase system using the so-called Ahh test [10, 14]. In this case, the recorded parameter is the rate of benzo(a)pyrene conversion into hydroxy derivatives, which after conjugation are eliminated from the cell. Apparently, inhibition of this process is undesirable for normal functioning of the body.

The change in the type and confirmation of the substituent at position C24 (the ethyl group instead of the methyl one in 28-homobrassinolide in contrast to 24-epibrassinolide) was detected only in the reaction with 7-ethoxyresorufin (Fig. 2). However, the effect, in this case, was weak and a twofold decrease in the reaction rate was not reached even when the concentration of 28-homobrassinolide in the reaction medium was increased to 250 μM. Nevertheless, it is worth mentioning that the direction of the effect is consistent with the data reported in [24], which showed a higher activity of the brassinosteroid with the ethyl substituent at position 24 as compared to the methyl one. At the same time, a strong inhibitory effect was observed in our experiments when the configuration of diol groups in the side chain of both compounds was changed from RR to SS. The IC_{50} value for (22S,23S-dihydroxy)-28-homobrassinolide (25 μM) determined in the reaction with 7-ethoxyresorufin was comparable to that reported for 28-homocastasterone (13 ± 2.6 μM) and castasterone (16 ± 5.3 μM), which showed maximum efficiency in experiments with human cancer cells [24].

It should be specially emphasized that the brassinosteroids used in this study had no significant effect on the other important function of the monooxygenase system, benzo(a)pyrene hydroxylation, which is required for its elimination from the body.

In general, the results of this study demonstrated the possibility of a direct, steroid receptor-independent effect of brassinosteroids on the enzymatic processes in mammals. The strength of this effect depends on the side chain structure, which suggests the possibility of a targeted modification of natural compounds to ensure required physiological effects.

REFERENCES

1. Khripach, V.A., Zhabinskii, V.N., and de Groot, A.E., *Ann. Botany*, 2000, vol. 86, pp. 441–447.
2. Khripach, V.A., Lakhvich, F.A., and Zhabinskii, V.N., *Brassinosteroidy* (Brassinosteroids), Minsk: Nauka Tekhnika, 1993.
3. Khripach, V.A., Zhabinskii, V.N., and de Groot, A.E., *Brassinosteroids: A New Class of Plant Hormones*, San Diego, CA: Academic, 1999.
4. Nemhauser, J.L. and Chory, J., *J. Exp. Bot.*, 2004, vol. 55, pp. 265–270.
5. Hu, Y., Bao, F., and Li, J., *Plant J.*, 2000, vol. 24, pp. 693–701.
6. Miyazawa, Y., Nakajima, N., Abe, T., Sakai, A., Fujioka, S., Kawano, S., Kuroiwa, T., and Yoshida, S., *J. Exp. Bot.*, 2003, vol. 54, pp. 2669–2678.
7. Brady, S.M.. and McCourt, P., *J. Plant Growth Regul.*, 2003, vol. 22, pp. 25–31.
8. Khripach, V.A., Zhabinskii, V.N., Ivanova, G.V., and Ol'khovik, V.K., *Vesti Akad. Nauk Belarusi, Ser. Khim.*, 1992, issue 1, pp. 70–72.
9. Akhrem, A.A., Lakhvich, F.A., Khripach, V.A., Kovganko, N.V., and Zhabinskii, V.N., *Dokl. Akad. Nauk SSSR,* 1985, vol. 283, pp. 130–133.
10. Lyakhovich, V.V. and Tsyrlov, I.B., *Induktsiya fermentov metabolizma ksenobiotikov* (Induction of Xenobiotic Metabolism Enzymes), Novosibirsk: Nauka, 1981.
11. Hoeven, T.A. and Coon, M.J., *J. Biol. Chem.*, 1974, vol. 249, no. 19, pp. 6302–6310.
12. Omura, T. and Sato, R., *J. Biol. Chem.*, 1964, vol. 239, no. 6, pp. 2379–2385.
13. Burke, M.D. and Mayer, R.T., *Chem. Biol. Interact.,* 1983, vol. 45, pp. 243–258.
14. Nebert, D.W. and Gelboin, H.V., *J. Biol. Chem.*, 1968, vol. 243, no. 23, pp. 6242–6249.
15. Turoverov, K.K. and Shchelchkov, B.V., *Biofizika*, 1970, no. 15, pp. 965–970.
16. Konev, S.V., *Elektronno-Vozbuzhdennoe Sostoyanie Biopolimerov* (Electronic Excited State of Biopolymers), Minsk: Nauka Tekhnika, 1965.
17. Wood, A.W., Wislocki, P.G., Chang, R.L., Levin, W., Lu, A.Y.H., Yagi, H., Hernandez, O., Jerina, D.M., and Conney, A.H., *Cancer Res.*, 1976, vol. 36, pp. 3358–3366.
18. Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., and Mayer, R.T., *Biochem. Pharmacol.*, 1985, vol. 34, pp. 3337–3345.
19. Dai, R., Zhai, S., Wei, X., Pincus, M.R., Friedman, F.K., and Vestal, R.E., *Drug Metabolism Disposit.*, 1998, vol. 34, pp. 989–992.
20. Brosa, C., *Critical Rev. Biochem. Molec. Biol.*, 1999, vol. 34, pp. 339–358.
21. Kubinyi, H. and Kehrhahn, O., *J. Med. Chem.*, 1976, vol. 19, pp. 578–587.
22. Yokoto, T., Morita, M., and Nakahashi, N., *Agric. Biol. Chem.*, 1983, vol. 47, pp. 2149–2157.
23. Takatsuto, S., Ikegawa, N., Mirishita, T., and Abe, H., *Chem. Pharm. Bull.*, 1987, vol. 35, pp. 211–217.
24. Malikova, J., Swaczynova, J., Kolar, Z., and Strnad, M., *Phytochemistry*, 2008, vol. 69, no. 2, pp. 418–426.
25. Behnia, K., Bhatia, S., Jastromb, B.S., Balis, U., Sullivan, S., Yarmush, M., and Toner, M., *Tiss. Engin.,* 2000, vol. 6, pp. 467–479.
26. Yu, LiJ., Matias, J., Scudiero, D.A., Hite, K.M., Monks, A., Sausville, E.A., and Waxman, D.J., *Drug Metabolism Disposit.*, 2001, vol. 29, pp. 304–312.

SPELL: 1. Lakhvich