ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2015, Vol. 41, No. 4, pp. 409–413. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © T.S. Frolova, T.P. Kukina, O.I. Sinitsyna, 2015, published in Bioorganicheskaya Khimiya, 2015, Vol. 41, No. 4, pp. 462–467.

# Genotoxic and Mutagenic Properties of Synthetic Betulinic Acid and Betulonic Acid

**T. S. Frolova[a, b, 1], T. P. Kukina[b], and O. I. Sinitsyna[c]**

[a] *Novosibirsk State University, Novosibirsk, 630090 Russia*

[b] *Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia*

[c] *Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia*

Received November 5, 2014; in final form, March 14, 2015

**Abstract**—Betulinic acid was synthesized from birch bark extract (Betula pendula), with betulonic acid being an intermediate of the synthesis. Both compounds were isolated with a purity of 95%. Genotoxicity and mutagenicity of the prepared compounds was analyzed by the Ames test and SOS chromotest and it was found that the substances show no mutagenic and genotoxic activity.

*Keywords: betulinic acid, betulonic acid, mutagenicity, genotoxicity, Ames test, SOS chromotest*

**DOI:** 10.1134/S1068162015040056

## INTRODUCTION

Triterpene compounds with a lupane skeleton are widespread secondary metabolites of various plant products [1]. As many native lupanes and their synthetic derivatives show different biological activities, they are of great interest in the preparation of valuable pharmaceuticals.

Betulinic acid is one of the most studied lupanes and may be isolated from plant raw materials by extraction. Thus, the yield of isolation of the target compound from birch bark (*Betula alba* L.) is 2.5% [2–4]. Synthesis of betulinic acid from betulin, the main component of the birch bark extracts of various species is more promising and the yield may be as high as 25% by weight of the birch bark [4]. Betulinic acid shows a number of valuable properties in biomedical tests, especially in experiments on the investigation of cytotoxicity against cancer cells [5–7]. Thus, the in vitro antitumor effect of betulinic acid in RKO and SW480 colon cancer cells and a significant in vivo inhibition of tumor growth in mice after oral administration of a drug containing betulinic acid has been observed [8]. The inhibition of prostate tumor growth has also been reported [9]. A combination of betulinic acid with radio- and chemotherapy has been shown in [10] to lead to a significant cytotoxic effect in glioma cells. The mechanism of this effect was supposed to consist in the ability of betulinic acid to trigger apoptosis through the release of mitochondrial apoptogenic factors [11]. It should be noted that normal cells were resistant to the toxic effect, that is, the effect was selective [12–15].

In addition to a significant antitumor effect, betulinic acid shows antioxidant properties [16, 17] and alcoholic extracts of plants containing betulinic acid also reveal the anti-inflammatory effect [18]. This compound was found in experiments on obesity in rats [19] to inhibit lipase activity and induce adipocyte lipolysis. The antimutagenic effect of extracts containing betulinic acid has also been observed [20].

Betulonic acid was considered for a long time only as a precursor of betulinic acid obtained in syntheses [21], however, recent studies have shown a number of valuable characteristics of betulonic acid such as antiviral, anticancer, antimicrobial, hepatoprotective, and immunostimulatory properties [22–27]. Investigation of the influence of derivatives of betulonic acid in combination with rimantadine on influenza virus has shown the possibility of reducing both side effects and dose of the drug due to the use of this combination [28].

Despite such a broad spectrum of valuable properties of betulinic acid and betulonic acid, the safety of their use in therapy has not been fully examined. According to the Bridges principles [29], the first stages of testing the safety of chemical compounds are rapid bacterial test systems, namely, the Ames test and SOS chromotest. Nevertheless, there are no data on testing betulinic acid and betulonic acid with the use of mentioned tests. Therefore, in the present work, betulinic acid was synthesized from betulin, one of the main components of the birch bark extract, to prepare the desired compound in an amount sufficient for microbiological tests. The samples of betulonic acid,

Abbreviations: NQO, 4-nitroquinolin-1-oxide; MTBE, methyl *tert*-butyl ether; *t*-$BuO_2H$, *tert*-butyl hydroperoxide.

[1] Corresponding author: e-mail: frolova@bionet.nsc.ru.

**Table 1.** Genotoxicity of betulinic acid and betulonic acid

| Compound (mM) | $I(c)_m \pm \sigma$ |
|---|---|
| DMSO | 1 |
| NQO (1.6) | $2.96 \pm 0.43$[a] |
| Betulonic acid (10) | $0.71 \pm 0.15$ |
| Betulinic acid (10) | $0.83 \pm 0.15$ |

[a] At the probability $P \geq 0.95$, the values are significantly different from those of the control.

**Table 2.** Genotoxicity of metabolites of betulinic acid and betulonic acid

| Compound (mM) | $I(c)_m \pm \sigma$ |
|---|---|
| DMSO | 1 |
| Benzo[α]pyrene (2) | $2.77 \pm 0.14$[a] |
| Betulonic $acid_{act}$ (10) | $1.15 \pm 0.12$ |
| Betulinic $acid_{act}$ (10) | $0.83 \pm 0.08$ |

[a] At the probability $P \geq 0.95$, the values are significantly different from those of the control.

which was an intermediate of the synthesis, were also examined.

## RESULTS AND DISCUSSION

### *Synthesis of Betulinic Acid*

Synthesis was performed via the described technique [4] with some modifications concerning purification of the target compound. The birch bark extract mainly composed of betulin and lupeol was used as an initial sample. In the first stage of synthesis, betulonic acid was prepared using Jones reagent (chromium(IV) trioxide in a mixture of dilute sulfuric acid and acetone), followed by reduction with sodium borohydride to yield betulinic acid. The target compounds were purified by recrystallization.

Measured melting points and $^1H$ and $^{13}C$ NMR spectra of betulonic acid and betulinic acid coincided with those reported in [30–32]. The prepared samples were shown by HPLC and LC-MS analysis to be characterized by a purity of 95%.

**Investigation of genotoxic properties of betulinic acid and betulonic acid by the SOS chromotest.** Cytotoxic properties of many compounds are based on their ability to induce DNA damages leading to prevention of replication [33]. The SOS chromotest was developed to detect these damages and determine their causative agents. The SOS chromotest is a quantitative colorimetric method for analyzing the activity of genotoxins. The test is based on the induction of SOS response of *E. coli* cells, with this response, in turn, inducing a number of functions as a reaction to DNA damage or termination of its synthesis. The structural gene of β-galactosidase (*lacZ*) being controlled by the promoter of DNA damage-inducible gene (*din*-gene), the SOS chromotest consists in the analysis of β-galactosidase activity and makes it possible to measure the ability of various physical and chemical mutagens to induce expression of *din*-genes in *E. coli* [34, 35]. In *E. coli* PQ37 strain (F–, *thr*, *leu*, *his-4*, *pyr*D, *thi*, *gal*E, *gal*K EIE *gal*T, *srl*300::Tn10, *rpoB*, *rpsL*, *urvA*, *rfa*, *trp*::*Muc*–, *sfiA*::*Mud*(*Ap*, *lac*), *cts*, *lac*ΔU169, PhoC) used in the SOS chromotest, the structural gene of β-galactosidase (*lacZ*) was introduced under the control of the promoter of *sfiA* din gene, so that β-galactosidase activity was dependent on the expression of this gene and induced under the influence of many genotoxic agents on the bacterial cells.

The analysis consisted in determination of the relative β-galactosidase activity in *E. coli* PQ37 cells induced by the influence of a supposed genotoxic factor. The analyzed compounds were tested by the conventional technique [36] with some modifications reported in [37].

Certain doses of potential genotoxic factors may inhibit the protein synthesis, thus, leading to incomplete determination of β-galactosidase induction. The ability of compounds to induce DNA damages resulting in prevention of replication is referred to as genotoxicity. In order to correct this influence, the synthesis of the total protein, β-galactosidase activity, and alkaline phosphatase activity (the latter is a constitutively expressed enzyme) was determined in the incubation period. The ratio of β-galactosidase activity to alkaline phosphatase activity obtained at the same exposure doses indicates the induction of *sfiA* gene even in the case of inhibition of the protein synthesis. The $I(c)$ induction factor of *lacZ* gene expression (induction of β-galactosidase activity) is the proportion of the ratio of β-galactosidase activity to alkaline phosphatase activity at a specified dose of a damage factor to the same ratio obtained in the absence of the factor. This value shows the magnitude of the damaging effect of a given agent.

The SOS chromotest was performed both with and without metabolic activation providing actually full spectrum of possible metabolites.

The values of β-galactosidase induction factor obtained after incubation of the test strain with betulinic acid, betulonic acid, and their metabolites are summarized in Tables 1 and 2. The studied compounds were used in the highest possible concentrations taking into account their solubility limits in DMSO.

Standard mutagens such as 4-nitroquinoline 1-oxide (NQO) and benzo[α]pyrene were used as positive controls in concentrations enough to induce an appreciable SOS response of *E. coli* PQ37 cells. It should be noted that there was no significant difference between the value of the induction factor obtained with the negative control and those obtained with betulinic acid and betulonic acid. Meanwhile, the reference NQO and benzo[α]pyrene showed high values of the induction factor (Tables 1 and 2).

**Investigation of mutagenic properties of betulinic acid and betulonic acid by the Ames test.** The standard test histidine-dependent *Salmonella typhimurium* TA 98 (hisD3052) and TA102 (hysG428) strains developed by Ames [38] and containing various mutations in the histidine operon [39–41] were used in the Ames test to detect the potential mutagenic activity of synthesized compounds [41]. The induction of mutations was tested by taking into account the reverse mutations from histidine auxotrophy to prototrophy resulting from a nitrogenous base substitution or reading frame shift caused by the influence of mutagenic factors. The base substitutions were detected by TA102 strain, while the reading frame shift was revealed by TA98 strain [39]. Thereby, the Ames test makes it possible to detect the mutagenic activity of various chemical compounds such as hydroperoxides, phenylhydrazine quinones, aldehydes, and other agents inducing base damages as well as the mutagenic activity of various radiations [40]. TA102 strain is a standard strain used to evaluate the mutagenic effect of oxidants.

As the SOS chromotest, the Ames test allows evaluating the direct mutagenic effect of a substance and its possible metabolites. The highest possible concentrations of studied compounds (in terms of their solubility limits in DMSO) were used.

The Ames test was performed via the original method [39]. The NQO and *t*-$BuO_2H$ mutagens and benzo[α]pyrene promutagen were used as standard mutagens. Data in Tables 3 and 4 show that the test *S. typhimurium* TA98 and TA102 strains being used, betulinic acid, betulonic acid, and their metabolites do not induce any base substitution mutations and frameshift mutations, whereas the standards show a pronounced mutagenic effects.

Thus, according to the Ames test and SOS chromotest, neither betulinic acid and betulonic acid nor their metabolites show noticeable genotoxic and mutagenic effects.

**Table 3.** Mutagenicity of betulinic acid and betulonic acid

| Compound (mM) | $N_m \pm \sigma$ | |
|---|---|---|
| | *S. typhimurium* TA98 | *S. typhimurium* TA102 |
| DMSO | 21 ± 4 | 322 ± 27 |
| NQO (1.6) | 93 ± 11* | – |
| *t*-$BuO_2H$ (0.011) | – | 760 ± 36[a] |
| Betulonic acid (10) | 18 ± 5 | 327 ± 28 |
| Betulinic acid (10) | 19 ± 4 | 3220 ± 16 |

[a] At the probability $P \geq 0.95$, the values are significantly different from those of the control.

**Table 4.** Mutagenicity of metabolites of betulinic acid and betulonic acid (*S. typhimurium* TA98 starin)

| Compound (mM) | $N_m \pm \sigma$ |
|---|---|
| DMSO | 60 ± 11 |
| Benzo[α]pyrene (2) | 154 ± 17[a] |
| Betulonic $acid_{act}$ (10) | 61 ± 10 |
| Betulinic $acid_{act}$ (10) | 56 ± 10 |

[a] At the probability $P \geq 0.95$, the values are significantly different from those of the control.

## EXPERIMENTAL

Chemicals of reagent grade (Russia) were used in the synthesis of betulinic acid and betulonic acid. NMR spectra of compounds dissolved in pyridine-$d_5$ were recorded on a AV-400 spectrometer at frequencies of 400.13 (for $^1H$ NMR) and 100.78 MHz (for $^{13}C$ NMR). The multiplicity of signals in $^{13}C$ NMR spectra was determined via the standard methods by experiments in *J*-modulation mode (JMOD) with off-resonance suppression. Melting points were measured with the use of a Kofler instrument. A Milichrom liquid chromatograph was used to perform analytical HPLC.

### *Preparation of Betulin Concentrate*

The bark of *Betula pendula* has been harvested in April 2012 near the city of Novosibirsk (Akademgorodok). The bark was air dried at room temperature and separated manually to obtain the outer bark and bast. The outer bark (100 g) was minced with scissors to obtain pieces of 1 × 5 mm size and extracted with methyl tert-butyl ether (MTBE) in a fluid percolator, with the extractant being preheated to 47°C. The solvent was added to a raw material so that the solvent layer was from 0.5 to 1.0 cm above the raw material and the ratio of the former to the latter was from 1.5 to 2. After one hour of infusion, the extract was drained through the bottom valve and a new portion of the solvent was added. The process was repeated four times to achieve the complete extraction of lipophilic compo-

nents and the combined ether extracts were evaporated to dryness on a rotary evaporator under reduced pressure (from 20 to 30 mmHg) provided by a water jet pump. The yield of extract was 38.4 g (38.4% by weight of the air-dry raw material). The content of betulin in the resulting extract determined by HPLC was 80.2%, that is, 30.8% by weight of the air-dry raw material.

### *Synthesis and Isolation of Betulinic Acid and Betulonic Acid*

The synthesis and isolation was performed by the previously proposed method [4]. The standard Jones reagent in acetone (5 mL) containing chromium trioxide was added dropwise to a stirred solution of 0.5 g of the birch bark extract (mainly composed of betulin and lupeol [2]) in acetone (20 mL) at 0°C. The mixture was stirred with a magnetic stirrer for 1 h, diluted with water to 80 mL, extracted with MTBE (4 × 20 mL) and the combined organic layers were extracted with 2% aqueous sodium hydroxide solution (3 × 20 mL) and washed with water (3 × 20 mL) to neutral reaction. The evaporated dry organic layer contained a lupenone concentrate. The combined aqueous extracts were acidified to pH 2 with 10% hydrochloric acid and extracted with hexane (2 × 20 mL) to remove aliphatic impurities, followed by extraction with MTBE (3 × 20 mL), washing the ether extract with water (3 × 20 mL) to neutral reaction, and evaporation to yield a concentrate of betulonic acid. Recrystallization from ethanol-hexane mixture resulted in 0.4 g of betulonic acid.

An alcoholic solution of potassium borohydride was added to a solution of 0.2 g of betulonic acid in 10% aqueous-alcoholic solution of potassium hydroxide (2 mL) at 8°C. The reaction mixture was stirred for 1 h, acidified with 5% hydrochloric acid and extracted with MTBE (3 × 20 mL). The resulting extract was washed with water (3 × 20 mL) to neutral reaction and evaporated to dryness to yield a concentrate of betulinic acid. Recrystallization from ethanol-hexane mixture resulted in 0.18 g of betulinic acid (28% of the initial raw material).

The standard mutagens such as NQO, *t*-$BuO_2H$, and benzo[α]pyrene, substrates such as *p*-nitrophenylphosphate and *o*-nitrophenyl-β-galactoside (Sigma), ampicillin (AppliChem, Germany), Bacto agar of special purity grade (Difco, USA), and other chemicals of reagent grade (Russia) were used in experiments. The induced rat liver S9 microsomal fraction was kindly provided by V.I. Kaledin (Institute of Cytology and Genetics).

Three bacterial strains were used in the work. *E. coli* PQ37 strain (*sfiA*::Mud(Ap *lac*) *cts*, *lac*ΔU169, *mal*$^+$, *uvrA, galE, galY*, PhoC, *rfa*) [35] provided by P. Quillardet (Institut Pasteur, France) was used in the SOS chromotest. *S. typhimurium* TA98 (*rfa*, Δ*uvrB*, +R) and TA102 (*rfa*, +, +R) strains [37] from the collection of B. Ames (University of California, USA) were employed in the Ames test.

### *SOS Chromotest*

The SOS chromotest was performed via the standard technique [34] with some modifications [37]. An overnight *E. coli* culture (PQ37 strain) was grown to an $OD_{550}$ of 0.45 ($2 \times 10^8$ cells/mL) with shaking at 37°C and diluted tenfold with LB medium. Aliquots (600 μL) of the suspension of bacterial cells were placed into a test-tube containing a solution (10 μL) of the test compound, with the exception being benzo[α]pyrene in the experiment with metabolic activation. The samples were incubated with shaking at 37°C for 2 h, followed by analysis of the enzyme activity.

In the test with metabolic activation, the bacterial culture was diluted tenfold with the activation mixture, β-galactosidase activity and alkaline phosphatase activity was then measured and the induction factor was calculated as the proportion of the ratio of β-galactosidase activity to alkaline phosphatase activity observed at the studied dose of a damage factor to the same ratio obtained in the absence of the factor. The SOS chromotest was carried out twice in triplicate and the result was shown as the $I(c)_m$ mean value of the induction factor calculated via the standard method [34].

### *Ames Test*

The Ames test was performed according to the method proposed in [39]. An overnight *S. typhimurium* culture (TA98 (*rfa,* Δ*uvrB*, +R) and TA102 (*rfa*, +, +R) strains) was grown to an $OD_{550}$ of 0.45 ($2 \times 10^8$ cells/mL) at 37°C for 16 h and the culture (100 μL) was placed into a test-tube containing a solution (10 μL) of the test compound. Two milliliters of 0.6% top agar containing histidine and biotin (for TA98 strain) or only histidine (for TA102 strain) were added, the mixture was thoroughly stirred by rotational movements of the test-tube between the hands and poured into Petri dishes containing 3% minimal glucose agar. In experiments with metabolic activation, a mixture (0.5 mL) of S9 was added to each 2 mL of top agar. The dishes were kept to dry the agar, thermostated at 37°C for 48 h and the colonies of revertants germinated into the underlying layer of the minimal glucose agar were counted. The Ames test was performed twice in triplicate and the result was shown as the $N_m$ mean value of the number of revertants per dish.

Statistical analysis was performed via the procedure described in [42]. The Student's *t*-test was used to determine the significant differences between the mean values of samples. The measurement error (σ) was determined as a standard deviation from the mean of independent measurements of the same sample [43].

## ACKNOWLEDGMENTS

The authors would like to thank V.I. Kaledin who kindly provided the induced rat liver S9 microsomal fraction.

## REFERENCES

1. Bishayee, A., Ahmed, S., Brankov, N., and Perloff, M., *Front Biosci*, 2011, vol. 16, pp. 980–996.
2. Tolstikov, G.A., Flekhter, O.B., Shul'ts, E.E., Baltina, L.A, and Tolstikov, A.G., *Khim. Interesah Ustoich. Razvit.*, 2005, no. 30, pp. 1–30.
3. Rzeski, W., Stepulak, A., Szymanski, M., Sifringer, M., Kaczor, J., Wejksza, K., Zdzisinska, B., and Kandefer-Szerszen, M., *Naunyn Schmiedebergs Arch. Pharmacol.*, 2006, vol. 374, pp. 11–20.
4. Yogeeswari, P. and Sriram, D., *Curr. Med. Chem.*, 2005, vol. 12, pp. 657–666.
5. Liu, E.H., Qi, L.W., Wu, Q., Peng, Y.B., and Li, P., *Mini Rev. Med. Chem.,* 2009, vol. 9, pp. 1547–1555.
6. Damle, A.A., Pawar, Y.P., and Narkar, A.A., *Indian J. Exp. Biol.*, 2013, vol. 51, pp. 458–491.
7. Micali, G., Lacarrubba, F., Bhatt, K., and Nasca, M.R., *Expert Rev. Anticancer Ther.*, 2013, vol. 12, pp. 9–21.
8. Chintharlapalli, S., Papineni, S., Lei, P., Pathi, S., and Safe, S., *BMC Cancer*, 2011, vol. 11, p. 371.
9. Chintharlapalli, S., Papineni, S., Ramaiah, S.K., and Safe, S., *Cancer Res.*, 2007, vol. 67, pp. 2816–2823.
10. Bache, M., Zschornak, M.P., Passin, S., Kessler, J., Wichmann, H., Kappler, M., Paschke, R., Kaluđerović, G.N., Kommera, H., Taubert, H., and Vordermark, D., *Radiat. Oncol.*, 2011, vol. 6, p. 111.
11. Fulda, S. and Debatin, K.M., *Med. Pediatr. Oncol.*, 2000, vol. 6, pp. 616–618.
12. Fulda, S. and Kroemer, G., *Drug Discov. Today*, 2009, vol. 14, pp. 17–18.
13. Petronelli, A., Pannitteri, G., and Testa, U., *Anticancer Drugs*, 2009, vol. 10, pp. 880–892.
14. Mullauer, F.B., Kessler, J.H., and Medema, J.P., *Anticancer Drugs*, 2010, vol. 21, pp. 215–227.
15. Reiner, T., Parrondo, R., Pozas, A., Palenzuela, D., and Perez-Stable, C., *PLoS One*, 2013, vol. 8, pp. 1–11.
16. Szuster-Cieseilska, A. and Kandefer-Szerseń, M., *Pharmacol. Rep.*, 2005, vol. 57, pp. 588–595.
17. Silva, M., David, J.P., Silva, L., Santos, R., David, J.M., Lima, L., and Fontana, R., *Molecules*, 2012, vol. 17, pp. 12197–12205.
18. Padilha, M.M., Vilela, F.C., Rocha, C.Q., Dias, M.J., Soncini, R., dos Santos, M.H., Alves-da-Silva, G., and Giusti-Paiva, A., *Phytother. Res.*, 2010, vol. 24, pp. 1496–1500.
19. Kim, J., Lee, Y.S., Kim, C.S., and Kim, J.S., *Phytother. Res.*, 2011, vol. 11, p. 3672.
20. Presnova, G.A., Chistyakov, A.N., and Durnev, A., *Bull. Exp. Biol. Med.*, 2004, vol. 138, pp. 475–478.
21. Melnikova, N., Burlova, I., Kiseleva, T., Klabukova, I., Gulenova, M., Kislitsin, A., Vasin, V., and Tanaseichuk, B., *Molecules*, 2012, vol. 17, pp. 11849–11863.
22. Tolstikova, T.G., Sorokina, I.V., Tolstikov, G.A., Tolstikov, A.G., and Flechter, O.B, *Russ. J. Bioorg. Chem.*, 2006, vol. 32, pp. 261–276.
23. Kislitsin, A.N., *Wood Chem.*, 1994, vol. 3, pp. 3–28.
24. Aiken, C. and Chen, C.H., *Trends Mol. Med.*, 2005, vol. 11, pp. 31–36.
25. Motteran, L., Pilone, M.S., Molla, G., Ghisla, S., and Pollegioni, L., *J. Biol. Chem.*, 2001, vol. 276, pp. 18024–18030.
26. Bastos, D.Z.L., Pimentel, I.C., De Jesus, D.A., and De Oliveira, B.H., *Phytochemistry*, 2007, vol. 68, pp. 834–839.
27. Pavlova, N.I., Savinova, O.V., Nikolaeva, S.N., Boreko, E.I., and Flekhter, O.B., *Fitoterapia*, 2003, vol. 74, pp. 489–492.
28. Savinova, O.V., Pavlova, N.I., and Boreko, E.I., *Antibiot. Khimioter.*, 2009, vol. 54, pp. 16–20.
29. Bridges, B.A., *Environ. Health Perspect.,* 1973, vol. 6, pp. 221–227.
30. Kogai, T.I., *Zh. Sibirsk. Feder. Univ.*, 2008, no. 1, pp. 97–104.
31. Kweyu, P.L., Bii, C., Akenga, A.T., and Wanjala, W.C., Antimicrobial marine natural products from the sponge, *Axinella infundibuliformis*, *Rec. Nat. Prod.*, 2008, vol. 2, pp. 116–127.
32. Lee, T.-H., Juang, S.H., Hsu, F.-L., and Wu, C.-Y., Triterpene acids from the leaves of *Planchonella duclitan* (Blanco) Bakhuizan, *J. Chinese Chem. Soc.*, 2005, vol. 52, pp. 1275–1280.
33. Escandani, M., Hamishenkar, H., Ezzati, Nazhad., and Dolatabadi, J., *Food Chem.*, 2014, vol. 153, pp. 315–320.
34. Quillardet, P. and Hofnung, M., *J. Bacteriol.*, 1984, vol. 157, pp. 35–38.
35. Quillardet, P., Huisman, O., D'Ari, R., and Hofnung, M., *Proc. Natl. Acad. Sci. U.S.A.*, 1982, vol. 79, pp. 5971–5975.
36. Quillardet, P. and Hofnung, M., *Mutat. Res.*, 1985, vol. 147, pp. 65–78.
37. Mersch-Sundermann, V., Kevekordes, S., and Mochayedi, S., *Mutat. Res.*, 1991, vol. 51, no. 60, pp. 51–50.
38. Ames, B.N., McCann, J., and Yamasaki, E., *Mutat. Res.*, 1975, vol. 31, pp. 347–364.
39. Maron, D.M. and Ames, B.N., *Mutat. Res.*, 1983, vol. 113, nos. 3–4, pp. 173–215.
40. Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A., and Ames, B.N., *Proc. Natl. Acad. Sci. U.S.A.*, 1982, vol. 79, pp. 7445–7449.
41. Levin, D.E., Hollstein, M., Christman, M.F., and Ames, B.N., *Methods Enzymol.*, 1984, vol. 105, pp. 249–254.
42. Vasil'eva, L.A., *Statisticheskie metody v biologii, meditsine i sel'skom khozyaistve* (Statistical Methods in Biology, Medicine, Agriculture, and Farming), Novosibirsk: Inst. Tsitol. Genet. SO RAN, 2007.
43. Hudson, D., *Statistika dlya fizikov* (Statistics for Physicists), Moscow: Mir, 1970.

*Translated by D. Lonshakov*