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Accessible Metabolites of Siberian Plants as a Source of Innovative Drugs

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Abstract

The paper presents the analysis of research insights into targeted functionalization of readily accessible metabolites from Siberian plants, such as lambertianic acid and betulin, and development of pharmacologically promising antitumour, hepatoprotective, neuroprotective, and analgetic agents based therefrom. The research was carried out by the staff of the Department of Medical Chemistry of NIOCH (Novosibirsk). Lately, birch and cedar have been widely used for the needs of the wood processing industry. Wastes generated from these manufacturing processes (birch bark and cedar needles) are a valuable source of bioactive substances to produce drugs and veterinary medicines. This determines the relevance of the development of environmentally friendly ("green") technologies for isolation of accessible metabolites, the procedure for their targeted chemical modifications, and pharmacological activity exploration with a view to developing innovative drugs.

Keywords: diterpenoids, lupane triterpenoids, betulin, bioactivity

INTRODUCTION

Natural compounds play a critical part during drug design and development. Speaking of drugs introduced into medical practice in 1981– 2014 (the total number of drugs of 1211), one may note that most of them are produced by organic synthesis (27 %). The second largest are derivatives of natural compounds including those obtained by combinatorial synthesis (21 %). A significant group is biological macromolecules (16 %). Synthetic drugs containing fragments (15 %) and pharmacophore groups of natural compounds (10 %) have been separated into separate groups. They are followed by vaccines (6%), pure natural compounds (4 %), and plant mixtures (1%) [1].

The current trends to generate accessible vegetable matter-based compounds point at the prospects of research aimed at the research of chemical properties and synthetic opportunities for plant di- and triterpenoids. Characterizing the biological activity of plant diterpenoids, in particular, furanolabdanoids covered in this review, one should note their anti-allergenic, neurotropic, anti-inflammatory, and antitumour properties [2]. Lupane triterpenoids, in particular, betulinic acid and its derivatives, are of great interest in the area of the development of antitumour agents [3].

Betulinic acid is cytotoxic to a number of tumour cells including cell lines of prostate, skin, lung, and liver cancers [4] and inhibits the growth, proliferation and breast cancer cell expansion, including tumour MCF-7, MDA-MB-231, MDA-MB-453, and BT474 cells lines and have an effect on cell division in the G1 phase and induces apoptosis with no effect on normal cells [5]. It refers to a class of mitokines, i.e. compounds that have an effect on mitochondrias in cells [4, 5]. However, low bioavailability and adverse adsorption parameters is a serious obstacle to the use of the substance in the clinic. In this regard, considerable attention is paid to the development of methods of modification of these metabolites (labdane diterpenoids and lupane triterpenoids) and their activity exploration. The present review involves analysis of some results acquired at the NIOCH SB RAS on the development of technologically simple methods of extraction and oxidation of betulin, synthesis of some nitrogen-containing derivatives of labdane and lupane terpenoids, and the investigation of their biological activity.

MATERIAL AND ENERGY SAVING TECHNOLOGIES FOR EXTRACTION AND OXIDATION OF BETTULIN

Birch bark is an accessible source of pentacyclic terpenoids, in particular, betulin (as high as 30 mass %) that is a valuable synthon to produce bioactive derivatives.

Most of the known works on birch bark extraction focused on improving the efficiency of extraction of valuable metabolites (betulin) using polar solvents, in particular, lower alcohols [6]. Other important aspects of extraction technology, such as the rational selection of extractors and their regeneration, are less explored. We researched opportunities to use medium polarity solvents for efficient extraction and transformations of triterpenoids.

It has been demonstrated that compared to the use of a traditional solvent, EtOH (95 %), during extraction of birch bark with a medium polarity solvent, EtOAc, or its mixture with aqueous EtOAc (96.5 %), extracts with very close betulin yields but lower contents of polar admixtures are produced [7].

We have explored issues related to extractant consumption and extraction energy. The amount of solvents retained on spent raw materials is 2.3–2.8 kg/kg (Fig. 1).

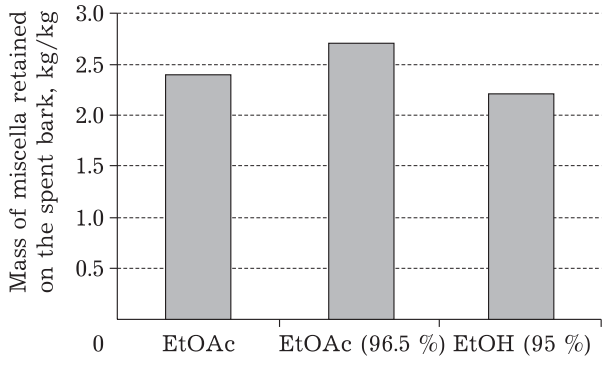


Fig. 1. Amount of the residual miscella (an extractant with dissolved extractives) retained by birch bark after extraction.

Solvent losses for spent raw materials per the yield of extract or betulin may reach ~ 6–9 kg/kg or ~10–15 kg/kg, correspondingly (20–30 % of the total extractant amount in the cycle).

The removal of residual solvents by extraction from spent raw materials is not efficient enough. After pressing on the hydraulic press (P = 100 kgf/cm^2), the residual content of EtOH and EtOAc over the bark was 0.6–0.8 kg/kg. The most complete regeneration of the solvent from raw materials is feasible by hydrodistillation.

We have considered two approaches to implementation of external birch bark extraction that are different in scale and the method of extractant regeneration from raw materials (Fig. 2).

It is possible to organize the periodic process of birch bark extraction with the regeneration of EtOAc from raw materials directly in the extraction apparatus by hydrodistillation on small and average scales [8]. Condensation of the distilled off azeotrope results in the formation of the organic phase (EtOAc, ~ 3.5 % of H_2O) and a small amount of the aqueous phase (~8.7 % of EtOAc). To avoid EtOAc losses, the separated aqueous phase is used in the following cycles of hydrodistillation. The removal with water vapour in order to remove the residual solvent is also used (Fig. 3).

The energy cost for the process is folded up from expenses of extraction (solvent and raw materials heating), for extract concentrating, and solvent regeneration from spent raw materials. To arrange a large-scale process, a variant with continuous extraction of birch

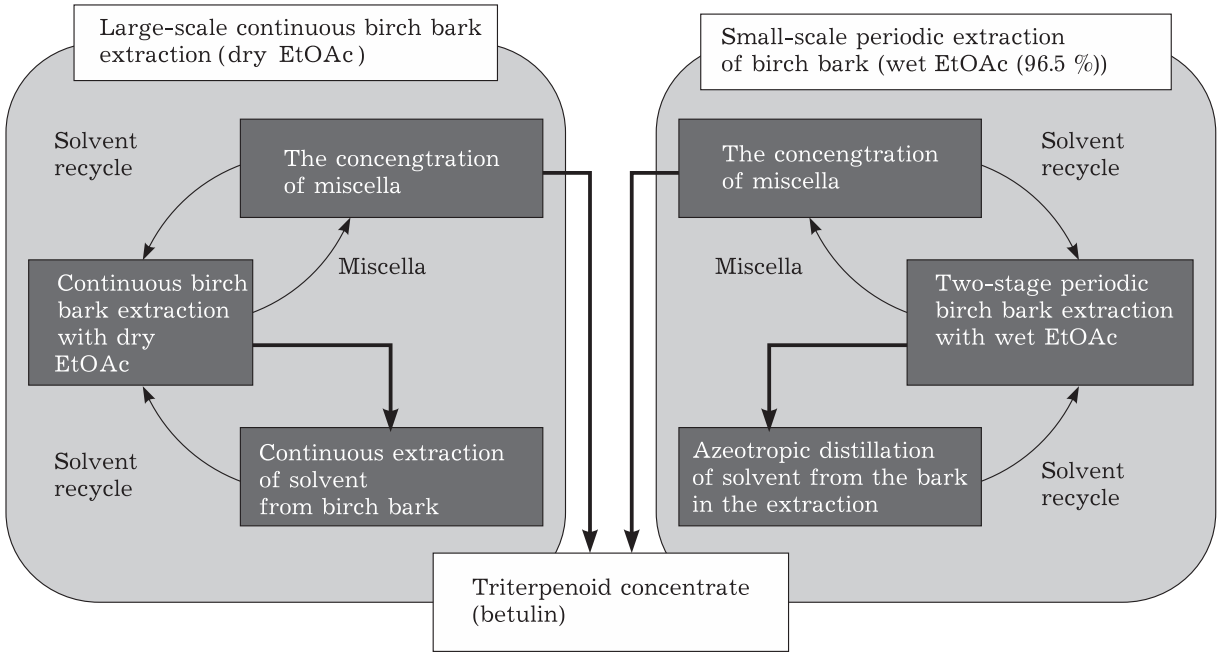


Fig. 2. Flow charts of extraction cycles with complete solvent recovery on large and small scales.

bark with hot EtOAc is probable. In such a case, regeneration from spent raw materials is possible in a continuously operating desolventiser device. Thus, specialized equipment and significantly higher costs for process organization are required. Figure 4 gives comparative analysis results for energy demands using various solvents and their complete regeneration. As can be seen from the data in Fig. 4, the main feature of energy demands is power consumption for concentrating the extract (1 miscella). Despite the fact that energy cost for extraction is slightly different for all compared extractants, power consumption for the regeneration of the residual extractant from raw materials is 1.5 times higher during extraction with 95 % EtOH than for dry EtOAc and ~1.1 times greater than in the process using wet ethyl acetate (96.5 %).

The case, when after extraction with EtOAc (95 %), the solvent is not removed from spent raw materials. As can be seen from the data presented in Fig. 4, even without considering the cost of solvent recovery from spent raw materials, in the process with EtOH (95 %), the total heat consumption is higher than that in schemes using EtOAc including solvent recovery from the extracted bark.

In this regard, the use of EtOAc that is a medium-polarity solvent to extract triterpenoids from birch bark is energy-efficient. Sterically hindered functional group-containing triterpenoid derivatives with the bulky carbon skeleton may be regarded as low-polarity compounds; therefore medium-polar compounds readily extract terpenoids from birch bark. Compared to alcohols, medium-polarity solvents extract lower amounts of polar impurities; lower energy costs are required for extractant regeneration. To arrange periodic processes of extraction and processing on a small and medium scale with a complete solvent recycle, it is possible to use standard non-specialized equipment.

Another example of material and energy-saving technology is the use of *t*-BuOMe, a medium polarity solvent, as the universal medium for extraction of birch bark and oxidation of betulin into betulonic acid [9]. As a rule, 5–7 types of solvents are used in the technological order for extraction and oxidation of betulin and purification of betulinic acid. Methyl tertiary butyl ether was selected as the universal solvent for extraction of birch bark and oxidation and separation of betulonic acid, as it efficiently dissolves betulin, poorly extract polar admixtures, and is low water-miscible [10]. Processes of neutralization of excess Cr^{6+}, separation of neutral mixtures after oxidation are also carried out in *t*-BuOMe.

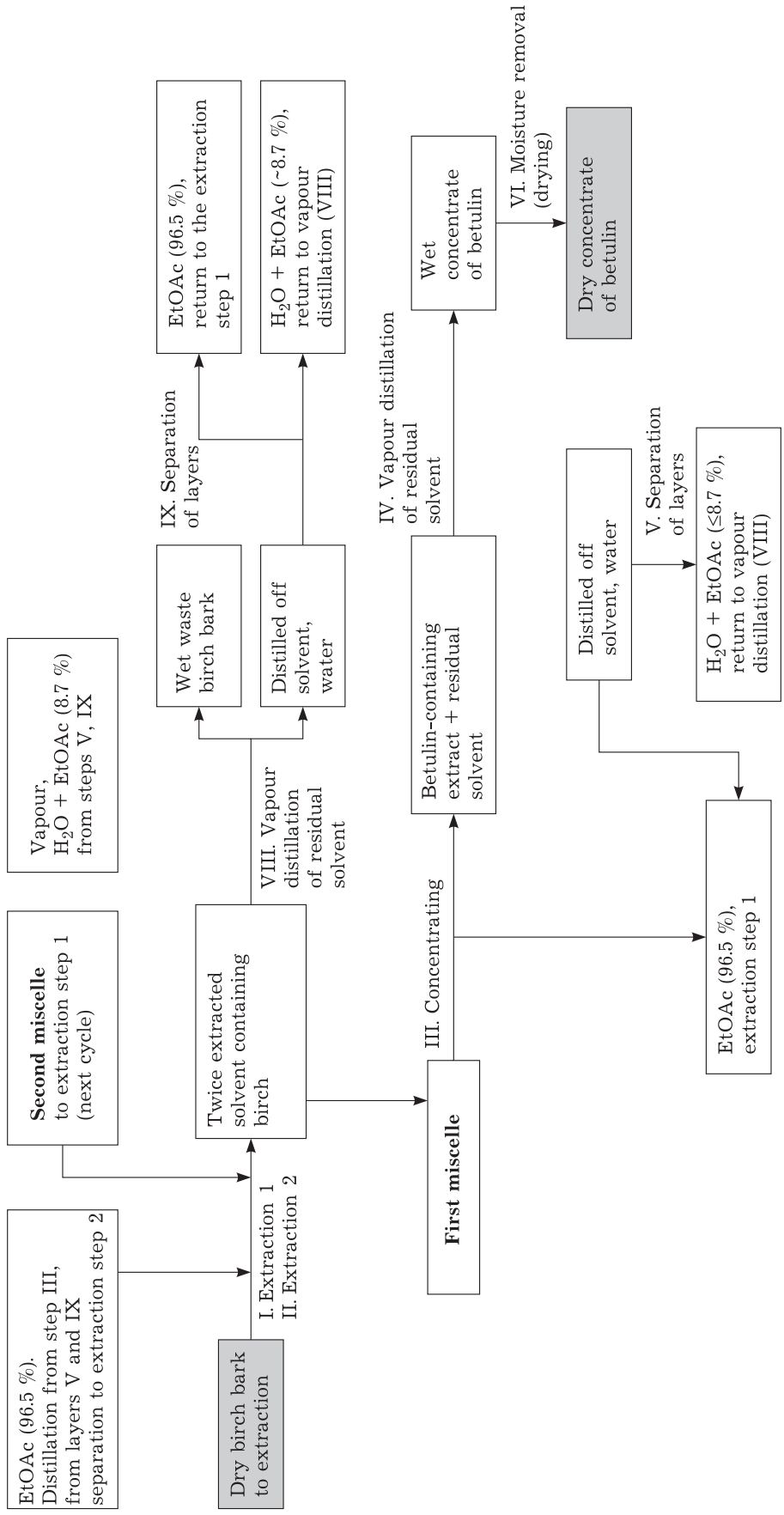


Fig. 3. Scheme for periodic two-step extraction of birch bark with complete extractant recycling by hydrodistillation (EtOAc 96.5 %): first and second miscella are merged extractant containing extractive matter after the first and second stages of extraction, respectively; I–IX are main operations on extract extraction and treatment.

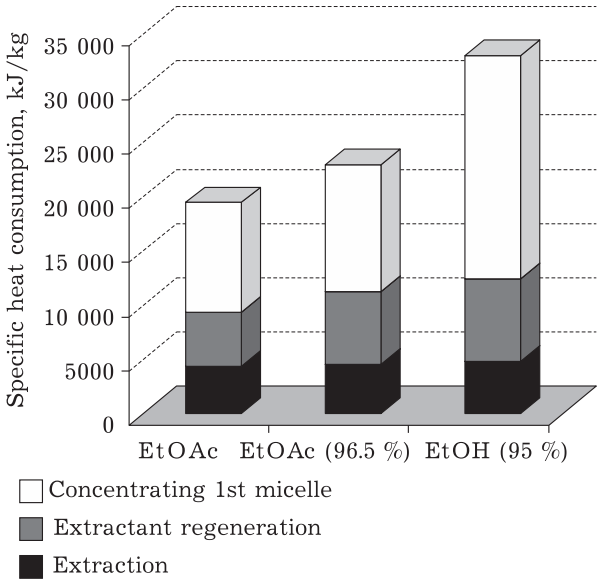


Fig. 4. Comparative assessment of specific heat consumption for birch bark extraction (kJ/kg extract) using different solvents: EtOAc, wet EtOAc (96.5.%), and ethanol (95 %).

Thus, the developed method guarantees multiple applications of a single *t*-BuOMe solvent in the extraction of the initial betulin, its oxidation and separation of neutral by-products (Fig. 5). The ether is regenerated and used in the next extraction cycles [11].

LABDANE DITERPENOID DERIVATIVES

Pinus sibirica diterpenoids

Lambertian acid **1** and its methyl ether **2** are among labdane diterpenoids accessible for modification. Being notable for significant accessibility from the turpentine [12], and also from needles and deciduous shoots of Siberian pine *Pinus sibirica* R. Mayr. [13], the specified compounds have valuable biological properties. In researching pharmacological activities of lambertianic acid, its antidepressant activity with a sedative component was revealed [14]. Reference [15] noted a significant potential of lambertianic acid **1** as an agent to treat allergies. The data have been acquired for its effect on the mediators of Allergy, including inhibition of products of interleukin-6 (IL-6), prostaglandin D_2 (PGD_2), of leukotriene C_4 (LTC_4), expression of cyclooxygenase-2 (COX-2) and the degranulation of β-hexosaminidase into PMA (phorbol ester). Properties of a stimulating antidepressant were revealed for lambertianic acid methyl ester **2** [16]. The accessibility of plant furanolabdanoids drives the interest to obtain other natural metabolites and virtually valuable substances based thereon.

Lambertianic acid **1** smoothly undergoes isomerization under the influence of *p*-toluenesulphonic acid in boiling benzene leading to flomisoic acid **3** [17]. Methyl lambertianate **2** also undergoes a similar transformation yielding flomisoic acid methyl ester **4** [18] (scheme 1).

Reference [19] describes handy synthesis the diterpenoid pinusolide **5** (Scheme 2). The synthesis involves oxidative methoxylation of methyl lambertianate **2** by the action of chloramine-B (or NBS) followed by treatment of diterpenoids 2.5-dimethoxytetrahydrofuran **6** with hydrochloric acid.

Diterpenoid pinusolide **5** was isolated from the turpentine of *Pinus sibirica* [20], and *Pinus koraiensis* [21], and also from the medicinal plant *Biota orientalis* [22, 23]. The chemopreventive and antileukemic potential of pinusolide **5** was investigated *in vitro* on cell lines BJAB of Burkitt's lymphoma [19]. It has been demonstrated that the pinusolide not only reduces the proliferative activity of tumour cells at relatively low concentrations but specifically induces apoptosis in 70 % of cells at a concen-

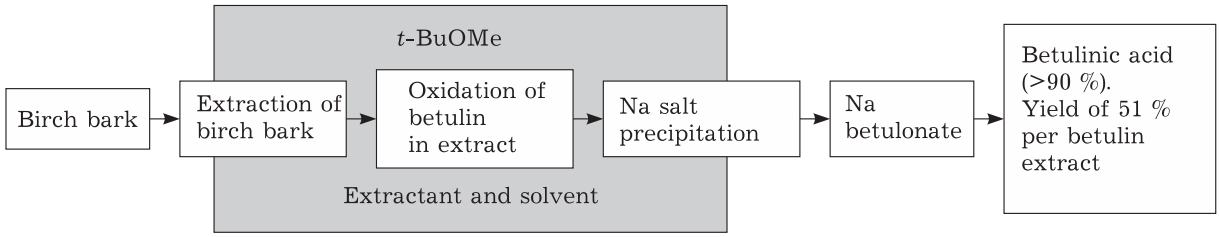
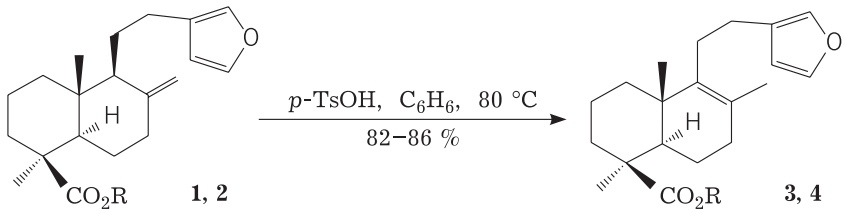
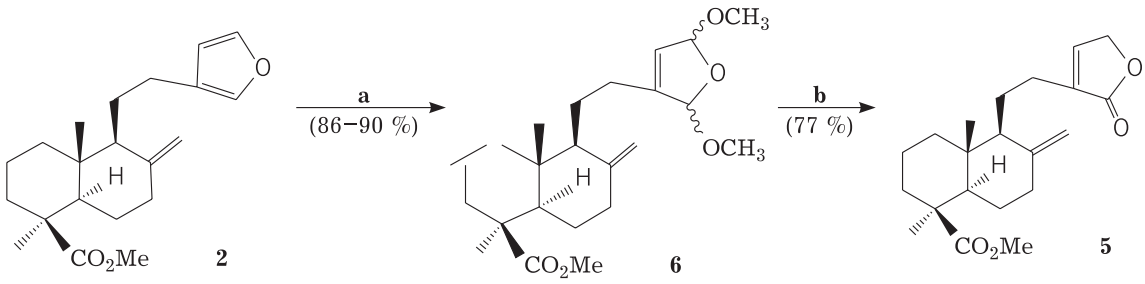


Fig. 5. Flowchart of extraction of birch bark t-BuOMe followed by oxidation of betulin and purification of betulonic acid through salt (purity of the resulting betulonic acid is over 90 %).



R = H(**1**, **3**); Me(**2**, **4**)

Scheme 1.



a: $PhSO_2NHCl$, MeOH or NBS, MeOH; **b**: HCl, 1,4-dioxane

Scheme 2.

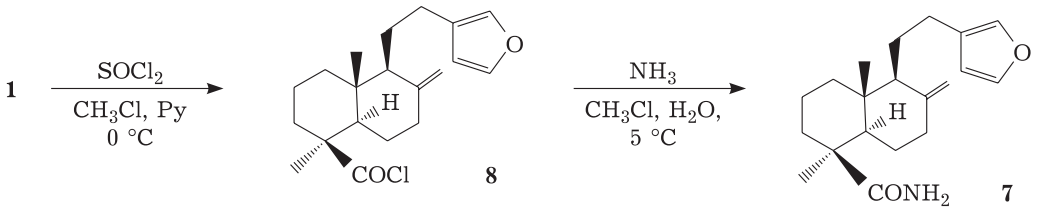
tration of 100 μmol/L. Apoptosis of BJAB cells (Berkitt lymphoma cell line) is mediated by the loss of mitochondrial membrane potential. In fact, pinusolide with a concentration of 100 μmol/L leads to a loss of mitochondrial membrane potential, which points out to the inner mitochondrial mechanism of apoptosis in the corresponding signalling pathway of cell death. There was significant induction of apoptosis by pinusolide (100 μmol/L) in an *ex vivo* experiment too. Herewith, DNA fragmentation occurred in both primary lymphoblastic cells and leukemic ones. Pinusolide *ex vivo* overcome the resistance to tetracycline and primary lymphoblasts derived in patients with high risk ALL (acute lymphoblastic leukemia) and poor response to chemotherapy [19]. Furthermore, it was found that pinusolide *ex vivo* overcame the resistance of the primary lymphoblasts derived in patients with high risk ALL (acute lymphoblastic leukemia) and poor response to chemotherapy [19]. The authors of [24] described pinusolide as a new antagonist of platelet aggregation factor. Neuroprotective activity of pinusolide **5** (from *Biota orientalis*) was investigated in [25].

Certain accessible derivatives of furanolabdanoids at the carboxyl functional group were synthesised. Lambertian acid amide **7** was obtained (with a yield of 91 %) by the interaction of lambertian acid **1** with thionyl chloride, followed by treatment of acid chloride 8 with aqueous ammonia (Scheme 3).

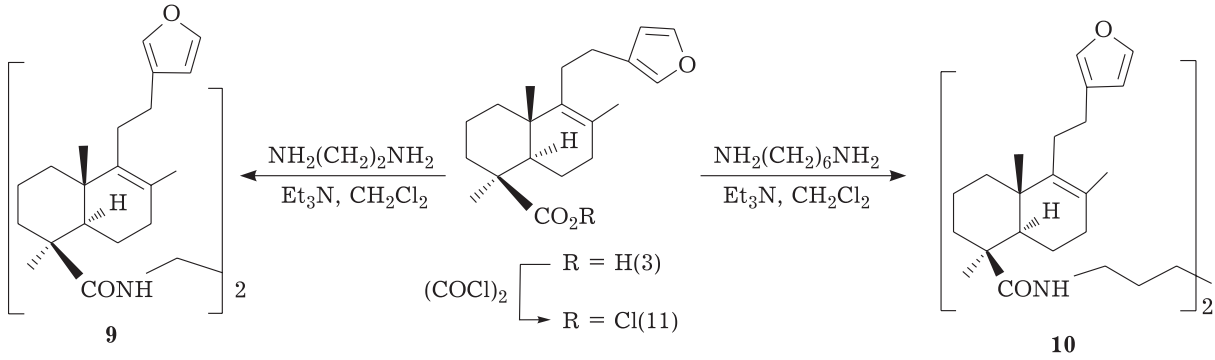
Based on flomisoic acid 3, N,N′-(ethane-1,2-diyl)- (9) and N,N′-(hexane-1,6-diyl)-bis-(labdatriene-4-carboxamides) (**10**) were synthesised (Scheme 4). Bis(labdatrienecarboxamides) **9** and **10** were obtained by condensation of flomisoic acid chloranhydride **11** with ethylenediamine or hexamethylene-1,6-diamine, respectively. Acid chloride 11 is quantitatively formed by treatment of compound **3** with oxalyl chloride in the presence of triethylamine in methylene chloride.

Biological activity of lambertianic acid amide (7), the mechanism of its action on brain structure and individual mediator systems

Analgesic activity. It is known that extracts of medicinal plants of the family Labiatae (Lamiaceae), *i.e. Phlomis* sp. and *Eremostachys* sp. containing glycosides of flomisoic acid **3**, show analgesic activity [26]. The presence of analgesic activity in the test "acetic cramps" *in vivo* was detected for plant diterpenoids marrubiin [27] and *Salvinorin A*, *i.e.* metabolite of the psychoactive plant *Salvia divinorum* [28]. This enhanced the production of research on the



Scheme 3.



Scheme 4.

biological activity of lambertianic **1** and flomisoic **3** acids, and also lambertianic acid amide **7**.

Analgesic activity was assessed by reducing pain response caused by chemical irritation of the peritoneum ("acetic cramps"), and also by the thermal sensitivity of animals (animal residence time on the hot plate) [29]. Table 1 gives the results. As can be seen, agent **7** shows a marked analgesic activity in the test "acetic cramps". This compound is a more effective pain inhibitor than analgin that is a more commonly used medicinal analgesic. According to analgesic activity, flomisoic acid **3** is comparable to analgin.

Compounds **1**, **3**, and **7** did not show a significant analgesic effect in the test of thermal sensitivity of animals. Thus, selective analgesic activities of lambertianic **7** and flomisoic acid **3** amides were educed on a visceral pain model.

Acute toxicity (LD_{50}) was determined by the Kerber method with a single intragastric administration to mice for compounds **1**, **3** and **7**, and also its **7** amide. The findings (see Table 1) make it possible to refer the compounds under study to the 3rd class (moderately toxic substances).

Changes in animal motion activity under the influence of lambertianic acid amide 7. The stimulating activity of lambertianic acid amide **7**

TABLE 1

Effect of labdanoids **1**, **3**, and **7** on pain reaction caused by thermal and chemical irritation

Group	Model			Acute toxicity (LD_{50}), mg/kg
	"Acetic cramps"		"Hot plate"	
	Number of cramps	RPR, %	Latent time of pain reaction, s	
Control	8.6±1.1	–	20.60±2.18	–
1	7.2±0.8	16.0	22.70±2.12	920
3	5.5±0.7*	35.9	24.80±3.45	633
7	4.2±0.7*	51.5	24.85±3.41	1500
Analgin	5.7±0.4*	33.6	20.2±2.26	–

Note. RPR is reducing pain reaction.

*$p < 0.05$.

was determined by animal behaviour research in the "open field" test [31]. Motion activity was assessed according to distance and rate parameters. Research activity was evaluated according to parameters of the number of vertical posts, the time in the vertical posts, the number and time of peepings into the holes. As it follows from the data of Table 2, compound **7** has a significant effect on the behaviour of animals in the "open field" test [30]. In intragastric administration at a dose of 5.0 mg/kg, the compound shows a marked stimulating effect; movement rate of animals and the distance travelled by them, and also the time of stay in vertical racks and the number of vertical racks increase. Herewith, the compound effect on animal behaviour is dose-dependent. When compound **7** is administered in a dose of 2.5 mg/kg, behavioural reactions of animals according to motor and research activity do not differ from the control. In a dose of 10.0 mg/kg, stimulating effect on motion activity parameters (distance and rate) becomes more apparent (in 1.2–1.3 times) than in a dose of 5.0 mg/kg. The total research activity of rodents with the administration of **7** in a dose of 10.0 mg/kg reliably increases twice compared to the control.

Table 3 gives the data on the ant anxiety activity of animals in the light-dark box test. The latter allows assessing the state of anxiety in animals, as animals with an increased level of anxiety prefer to be in a dark chamber, and with low – in the light compartment of the box. Diazepam in a dose of 2.5 mg/kg was used as a comparison drug. Its administration resulted in a statistically significant increase in both indicators. As it follows from the findings, lambertian acid amide **7**, unlike diazepam, does not have an effect on the latent time of the first entry of the animal to the dark chamber, nor the time of its presence in the dark chamber. These results make it possible to conclude that administration of compound **7** does not result in enhanced anxiety level and, therefore, the detected ability to increase animal research activities in the "open field" test is probably related to its ability to provide a general stimulating effect.

It is known that the stimulatory effects of agents are implemented through various neurotransmitter systems. Chloral hydrate sleep test is used to explore the *in vivo* effect of compounds on glutamate and the GABA-ergic system [31]. The effect of lambertianic acid amide **7** was investigated according to the influence on the hypnotic effect of chloral hydrate (Table 4). It was administered intraperitoneally in the amount of 350 mg/kg 1 h after the introduction of the agent. The latent time of sleep and animal sleep duration were assessed. As demonstrated by research, amide **7** with a dose of 5 mg/kg does not change the latent time of sleep of animals, while diazepam significantly reduces it. Compound **7** almost does not have an effect on the duration of chloral hydrate sleep; diazepam increases it twice.

Thus, based on the research on behavioural reactions of animals in the open field and dark-light box tests, and also according to the effect of chloral hydrate on the hypnotic effect, it was found that lambertian acid amides (see Scheme 1, compounds **1**, **2**) had a general marked stimulating effect.

Research on modes of action of lambertianic acid amide against the effect of social

TABLE 2

Effect of lambertian acid amide **7** on animal behaviour in the open field test (intragastric injection)

Доза **7**, mg/kg	Parameter							
	Distance, cm	Rate, cm/s	Number of vertical racks	Time of vertical racks, s	Number of peepings	Time of peepings, s	Number of hops	Research activity
Контроль	268.0±18.4	2.2±0.2	7.0±1.9	10.1±3.0	4.1±1.1	5.5±1.6	0.6±0.4	11.1±2.6
2.5	264.6±19.3	2.2±0.2	6.4±1.4	9.5±2.1	4.5±1.1	5.8±1.0*	0.8±0.2	10.9±1.5
5.0	328.3±21.6*	2.7±0.2*	12.0±2.1*	18.5±3.1**	6.0±1.0*	7.4±1.3**	0.8±0.6	18.0±1.9**
10.0	379.3±23.1#	3.1±0.2**	14.3±0.7**	21.1±2.8#	6.1±0.4	7.8±0.6	1.3±0.4*	21.4±1.9#

*$p < 0.1$; **$p < 0.05$; #$p < 0.01$ with regard to control.

TABLE 3

Effect of lambertian acid amide **7** on animal behaviour in the light-dark box test

Group	Parameter	
	Latent time of entering dark box compartment, s	Time spent in dark box, s
Control	58.28±9.18	93.21±6.64
7	48.72±6.12*	85.50±7.35*
Diazepam	132.00±22.70**	159.87±11.48#

*$p < 0.1$; **$p < 0.05$; #$p < 0.01$ with regard to control.

stress. We found that lambertian acid amide **7** had an apparent stress-protector effect; increased communicativeness and animal motion activity, and also decreased paranephros hypertrophy under conditions of social discomfort of female mice (living in a cage with an aggressive male placed behind a transparent perforated partition, and daily presence in intermale confrontations).

The experiments performed on hippocampus sections involved the determination of the ability of amide **7** to maintain functioning the NMDA-receptor-channel complex in the absence of the endogenous ligand (magnesium ions) and the research on the NMDAR-dependent synaptic potential under physiological conditions. It was demonstrated in the experiments that amide **7** might have an effect directly on nerve tissue (hippocampus sections). The apparent neuroprotective effect was revealed [33].

Thus, based on the totality of the revealed effects and moderate acute toxicity of lambertianic acid amide **7** (LD_{50} 1500 mg/kg according to the Kerber method) may be recommended for in-depth preclinical research as a neuroprotector.

Antitumour activity of N,N'-(alkanediyl)bis[labda-7(9), 13,14-trien-4-carboxamides]

Plant terpenoids attract attention as inhibitors of tissue invasion and inducers of tumour cell apoptosis, which makes it promising to search for cytostatic agents for anticancer therapy in this series [34]. Apart from the characterization of the anticancer potential of the compounds-leader pinusolide **5** [19], we obtained the data on the cytotoxic and antitumour activity of bis(labdacarboxamides) **9** and **10**. Cytotoxic activity of the compounds was explored according to the ability to inhibit tumour cell growth in cultures: MT-4 (T-cell leukemia lymphocytes), BT-474 with high expression of HER-2 (breast cancer), MDA-MB-231 and MCF-7 with low expression of HER-2 (breast cancer), melanoma cells MEL-8 (Table 5). The standard MTT test was used to determine GI50 [35]. It was found that compound **10** had the highest cytotoxic activity for melanoma tumour cells MEL-8 (4.16 μmol/L) and B-474(5.33μmol/L).Bis(labdarienecarboxamide) is a **10** 5 times more active inhibitor of the viability of tumour cells MEL-8 compared to compound **9** and is 8–10 times compared to flomisoic acid **3**. Compound **10** shows inhibito-

TABLE 4

Effect of compound **7** on chloral hydrate sleeping time

Group	Parameter	
	Latent time to falling asleep, s	Sleep duration, min
Control	240.62±13.57	86.77±8.74
7	266.25±17.84*	105.76±10.38**
Diazepam	192.50±18.02**	207.79±6.19#

*$p < 0.1$; **$p < 0.05$; #$p < 0.01$ with regard to control.

ry activity for T-cell leukaemia MT-4 cells (7.17 μmol/L) and is a 5–7 times more active inhibitor of the viability of these cells than compound **9**. Herewith, there was selectivity against tumour cells BT-474 for **9**. According to cytotoxic concentration on tumour cells of melanoma MEL8 and the cancerous mammary gland MDA-MB-231, compound **10** is comparable to doxorubicin [36].

Antitumour effects of bis-labdanoids **9** and **10** were *in vivo* investigated for CBA-line mice with a mass of 25–30 g. The mice were transplanted intramuscularly with cells of malignant murine lymphoma RLS resistant to cyclophosphane (500 thousand cells). This strain of the lymphoma grows as a solid lump and is characterised by a quick progressive growth. Investigated compounds **9** and **10** were administered in the course mode intragastrically, beginning from the fifth day after the mode of four times a day with a dose of 100 mg/kg as a suspension in water with Twin-80 (total course dose of 400 mg/kg). The effect of a complex of cytostatic drugs administered once to the reference group of mice according to the standard scheme of CHOP polychemotherapy (PCT) was a standard of antitumour effects: cyclophosphane (50 mg/kg), doxorubicin (4 mg/kg), vincristine (0.1 mg/kg) and prednisolone (5 mg/kg). Animals with tumour without treatment were the control. The dimensions of tumour nodes were measured in dynamics at the stage of progressive tumour growth. Table 6 gives the results of measurements of tumour size during the period of agent administration [36]. The antitumour effect was assessed according to tumour growth inhibition (TGI) index, which was defined as the difference between the average tumour size in the control and experimental groups related to the average tumour size in the control group. It was found that in the early period of observation (13th day after the vaccination), agents **9** and **10** were not inferior to the PCT in antitumour effect, and at the end of this period (17th day), both agents were superior to the effect of anticancer chemotherapy (see Table. 6). Herewith, bis(amidolabdanoid) **10** with a linker chain of six carbon atoms demonstrated the highest and significant effect.

An additional experiment on mice that were implanted with the above tumour researched the antitumour effects of compound **10** compared to paclitaxel. Agent **10** was administered intragastrically as a solution in sunflower oil with a dose of 100 mg/kg three times a day (course dose of 300 mg/kg, two days after the inoculation). The reference group was administered with an aqueous solution of paclitaxel with a dose of 30 mg/kg, and the control one – with sunflower oil in an amount of 100 mg/kg. Tumour size was determined two days after the withdrawal of administration of compounds or from the 11th to the 18th day after the vaccination (before the beginning of the death of animals in groups). Table 7 gives research results. It was found that compound **10** had antitumour effects, which were expressed in a significant growth delay, while the effect was not inferior to paclitaxel but had a shorter duration of the effect after the withdrawal of administration (on the 18th day, unreliable data) [36].

Thus, bis-labdanoids **9** and **10** have antitumour effects during intragastric administration to mice. The latter are expressed in the delay of malignant tumour growth. Herewith, compound **9** is not inferior to, and **10** is superior to cytostatic polychemotherapy (PCT) in the efficiency according to the standard SNOR scheme (cyclophosphane, pharmarubicin, vincristine, and prednisolone). The results achieved

TABLE 5

Cytotoxic activity of bis(labdatrienecarboxamide) derivatives **9** and **10** against human tumour cell lines

Compounds	GI_{50}, μM			
	MT-4	MEL-8	MDA-MB-231	BT-474
Doxorubicin	2.81±0.82	5.12±0.51	7.91±0.54	3.47±0.08
Flomisoic acid **3**	24.51±2.25	37.11±3.55	27.76±2.51	n/d
N,N′-(ethane-1,2-diyl) bis(labdtriene-4-carboxamide) **9**	41.36±5.44	21.08±3.23	16.33±1.98	5.77±1.09
N,N′-(hexane-1,6-diyl)bis(labdatiene-4- carboxamide) **10**	7.17±1.19	4.16±0.39	9.87±1.84	5.33±0.86

Note. 1. GI_{50} concentration of the substance at which 50 % inhibition of tumour cell growth was observed after 72 hours of incubation. 2. N/d means no data available.

TABLE 6

Changes in dimensions of RLS lymphoma transplants in mice during intragastric administration of agents **9**, **10**

Group	Tumour dimensions, cm^3			
	Time, days			
	10	13	15	17
Control	0.61±0.05	1.31±0.07	1.89±0.16	3.14±0.29
CHOP PCT	0.51±0.06	0.79±0.08**	1.51±0.04*	2.58±0.14*
9	0.58±0.09	0.83±0.19	1.48±0.33	1.81±0.55*
10	0.46±0.06	0.66±0.13#	1.07±0.26*	1.68±0.44*

Note. PCT is polychemotherapy.

*$p < 0.05$, **$p < 0.01$ significant differences from control.

#$p < 0.001$ significant differences from PCT.

for the transplantable lymphoma resistant to cyclophosphane show the potential ability of these bis-labdanoids to overcome the phenomenon of drug resistance of tumour strains.

TRITERPENOIDS OF THE LUPANE SERIES

Betulin **11** (Fig. 6) is a highly promising basic compound. New selectively acting medicinal agents with higher biological potential and higher hydrophilicity compared to the prototype (the compound leader), betulinic acid **12** were produced based thereon. Several reviews discussed opportunities of structural modifications of betulin at different positions of the lupane skeleton without changing the carbon framework [37, 38]. Significant attention is paid to the synthesis of betulin derivatives under metallocomplex catalysis conditions [39, 40]. A large data set was acquired thus far for the structure versus antitumour activity of derivatives of betulin and betulinic acid [41]. Attention was paid to opportunities of the introduction of nitrogen-containing heterocyclic substituents at the isopropyl group [42] and in the position C-30 of the lupane skeleton [43]. We synthesized of a wide range of betulonic acid **13** amides by the interaction of betulonic acid chloroanhydride with different amines [44, 45].

The selection of amines was due to their availability and an opportunity to produce products with selective pharmacological action (the use of pharmacophore amines). Not only the revealed regularities of the investigated reactions but also the synthesized compounds themselves may be significant as new bioactive agents. In a series of C-30 triazolyl-substituted lupane derivatives, promising cytotoxic agents were found. Figure 6 gives the structures of the most promising compounds of betulonic acid α-alanineamide **14** and betulonic acid β-alanineamide **15**.

TABLE 7

Change in dynamics of the growth of RLS lymphoma transplants in mice after intragastric administration of agent **10** or paclitaxel

Group	Tumour dimensions, cm^3			
	Time, days			
	11	13	15	18
Contro (sunflower oil)	0.42±0.03	1.38±0.13	2.64±0.27	4.41±0.30
Paclitaxel, 30 mg/kg	0.40±0.05	1.10±0.15	1.85±0.17*	3.28±0.29*
10, 100 mg/kg	0.30±0.04*	0.83±0.08**	1.96±0.14*	4.00±2.00

*$p < 0.05$, **$p < 0.01$ significant differences from control.

Research on pharmacological properties of betulonic acid β-alanineamide 15

Betamide is a derivative of betulonic acid containing the amino acid residue of β-alanine **15** in position C-28 of the side chain refers to low-toxic substances (LD_{50} over 5000 mg/kg, single intragastric administration), does not have a locally irritating allergic mutagenic and embryotoxic effects, does not exert a cumulative impact.

Protective and antiproliferative properties of betamide were researched in mice and rats exposed to cytotoxic drugs, modelling poly- and monotherapy of malignant tumours. Antitumour and antimetastatic activities were determined both in isolated mode of introduction of the agent and in the context of SNOR (PCT) adapted for laboratory animals with a single intraperitoneal simultaneous introduction of a set of cytotoxic drugs in doses of 1/5 LD_{50}, in mice of the lines CBA/Lac and C57BL/6 with transplantable tumours (lymphoma, LS, RLS lymphoma, and Lewis lung carcinoma). An opportunity to potentiate the therapeutic effect and reduce organotoxic effects of cytostatic drugs used in tumour chemotherapy was determined in the same experiments.

Betamide was singly administered intragastrically (a dose of 500–1000 mg/kg) or for 8 days (a dose of 50 mg/kg) in an isolated mode of administration, once the dimensions of the primary tumour node reached 1 cm^3. To assess the antimetastatic effect, the number of metastatic nodes in the lungs and liver of mice were calculated. It has been found that betamide delays primary node growth by 20–35 %, and also reduce the number of metastatic nodes in the lungs and liver by 50–70 % and 40–50 %, respectively [46, 47].

As demonstrated by experiments on CBA/Lac mice with RLS lymphoma received SNOR (PCT), the combined use of betamide reduced the amount of necrotized nephrocytes by 77 %, increased the number of cells with mild hyaline-drop dystrophy by 53 % and reduced the lumen of proximal tubules by 43 % compared to the PCT group [48]. The introduction of the agent against the PCT into the liver of these same mice resulted in a decrease of the bulk density of necrosis zones by 63 % and an increase in areas of dystrophic modified hepatocytes by 19 %. Herewith, the degree of dystrophy decreased from hydropic and balloon to weakly expressed hyaline-drip, the volume density of sinusoids in-

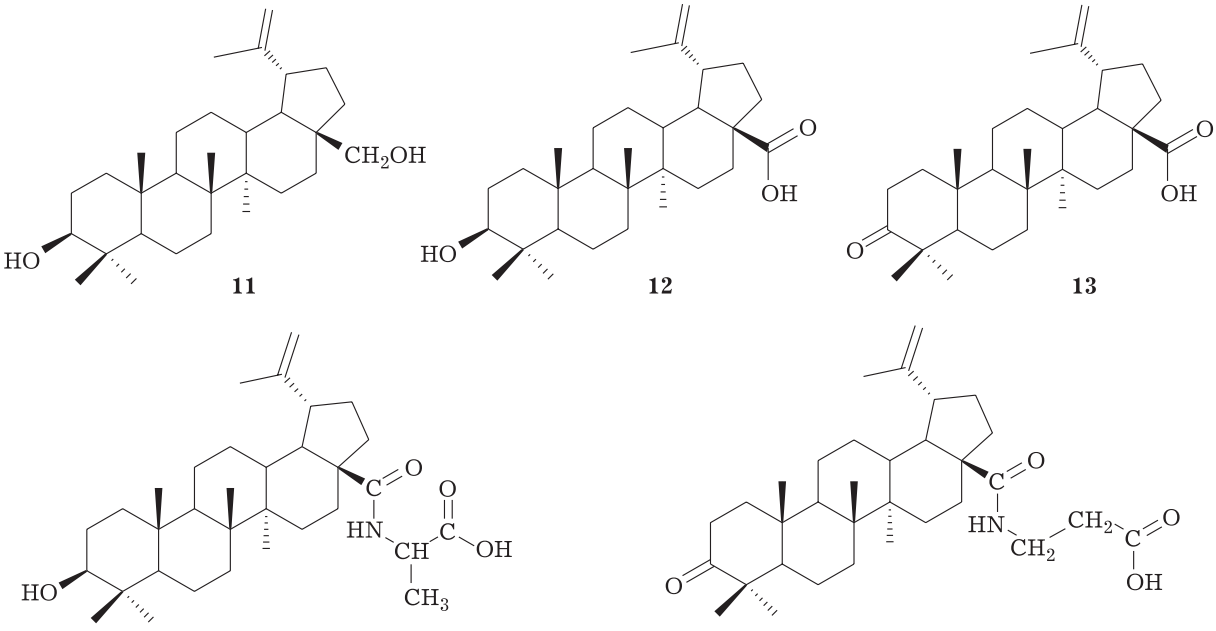


Fig. 6. Betulin **11**, betulinic **12** and betulonic **13** acids, lupane amide derivatives.

creased (28 %), intrahepatic cholestasis decreased, and the amount of glycogen in all hepatocytes rose [49].

In the liver of C57Bl/6 mice with Lewis lung carcinoma, betamide administration against the PCT caused a decrease in monocellular necrosis by 53 % and a decrease in the severity of dystrophy, increased the volume density of sinusoids by 33-40 %, and reduced the number of metastases therein compared with PCT introduction only [50]. In the kidneys of the same mice, under the influence of triterpenoid administration against the PCT, there was a high nephroprotective effect (by 75 %) in relation to the group receiving only the PCT [51].

Betamide administration to healthy Wistar rats for 14 days in a dose of 50 mg/kg against single dosing according to the CHOP PCT scheme reduced the number of hepatocytes with dystrophic changes and necrotized cells by 75–90 % and 35–45 %, respectively. In the kidneys, the amide significantly weakened the inflammatory effect of cytostatics, decreased the fraction of nephrocytes of proximal tubules with hydropic degeneration and that of necrotic changes by 65–72 % and 60 %, respectively. In the thymus, there was the protective effect of betamide that became apparent in an increase of the cortical-brain index and a decrease in the number of Gassal bodies in the portion of the brain [52-54]. There were the thymus protective effects of the amide against single administration of cyclophosphane to healthy rats in a toxic dose of 125 mg/kg that caused persistent depressive disorder of blood-forming organs. There was a trend towards the more rapid restoration of thymus mass in combined administration of betamide and doxorubicin. Furthermore, it was demonstrated that the triterpenoid exerted lipid profile correction, mainly by a reduction of the level of low-density lipoproteides [55].

As demonstrated by analysis of the morphological picture in the developed stage of the disease of tumour-bearing mice, betamide retains a modulating effect on the total number of leukocyte cells under PCT conditions, herewith, it has a stimulating effect on monocytes.

Agent administration against the PCT causes a meaningful increase in the number of mature leukocytes and enhances generating non-mature myelocytes in animals with the Lewis lung carcinoma. It is worth noting that betamide credibly increases maturation indices of erythrocytes and granulocytes, which may be considered as its stimulating effect on the differentiation of these cell elements of hematopoiesis. These maturation indices of bone marrow cells in mice with RLS lymphoma exposed to cytostatics and triterpenoids prove the stimulating effect of betamide onto granulocytic and monocytic blood sprout. There were similar but less apparent shifts in the dynamics of blood neutrophils and monocytes in betamide administration against single dosing doxorubicin, whereat there was also a trend to normalizing white blood indicators, which correlated with the results acquired in the experiment with cyclophosphane. The findings suggest hematoprotective effects of betamide under conditions of exposure to cytostatics. Herewith, the corrective effects of the agents are mainly directed at cells of the granulocytic and monocytic series being the effector in the inflammatory and immune responses.

There was a dramatic attenuation of the intensity of peroxide processes in mice with transplantable tumours received the PCT under the influence of betamide. The blood concentration of the secondary product of peroxidation, *i.e.* malonic dialdehyde, reduced by 72–75 % at Lewis lung carcinoma and by 40 % at RLS lymphoma compared to animals received only cytostatics.

As demonstrated in experiments on Wistar rats, betamide increased the activity of cytochromes P450 – P450IIC, P450IIIA4 P450IIE1 determinable according to substrate utilization rates (accordingly, amidopyrine, erythromycin, and aniline, respectively). Amide administration already in 6 h enhanced amidopyrine, erythromycin, and aniline metabolism in 1.81, 1.57, and 1.65 times, respectively. Agent course administration against CHOP PCT restored cytochrome P450IIIA4 activity in 6 days after exposure and increased P450IIE1 activity by 26.5 and 24.1 %, respectively, on the 7th and 14th days of observation [56].

Hepatoprotective properties of betamide were also researched in a model of chronic toxic hepatitis complicated with fibrosis and cirrhosis. Toxic liver damage was simulated by 6-week intragastric administration of tetrachloromethane and ethanol. At this background, course administration of betamide in a dose of 50 mg/kg completely prevented the

development of toxemia and fibrosis in all steps of research. Herewith, there was stimulation of regenerative-plastic processes, relief of cholestasis and cytolysis, and also a reduction of anoxic damage of hepatocytes in liver tissue [57–59].

There was carried out research on the long-term 8-week administration of betulin in a dose of 50 mg/kg against developing experimental chronical gastritis. The latter was induced by intragastric administration of a 2 % solution of sodium salicylate in ethanol. As demonstrated by research results, betamide efficiently reduces signs of chronic atrophic gastritis; decreases inflammation severity and regenerates the gastric mucosa due to the restoration of a pool of HCl-secreting cells and those that secrete protective slime [60].

As demonstrated in an experimental model of osteomyelitis induced with *Staphylococcus aureus* inoculation into the tissue of shin bone of male rats, betamide has an apparent anti-inflammatory effect. According to immunohistochemical and morphological research data for bone tissue, intragastric administration of betamide for 3 months in a daily dose of 100 mg/kg prevents generalization and chronization of the inflammatory process (the formation of extensive areas of necrosis and infiltration, osteoclastic bone resorption, and generating sequesters) and facilitates its resolution. It has been found that there is the earlier and more apparent formation of loose fibrous connective and coarse-fibrous bone tissue under the influence of betamide. The latter causes accelerated onset of the phase of functional adaptation contributing to the fast end of regenerative endochondral osteohistogenesis with generating mature bone tissue [61].

The presented results attest to the presence of antioxidant, hepatoprotective, anti-inflammatory, immunomodulatory, antitumour, and antimetastatic activities in betamide. Herewith, betamide is a low-toxic compound. When introduced to animals with transplantable tumours combined with anticancer drugs, betamide normalizes the amount of blood leukocytes, accelerates leukocyte maturation in bone marrow, normalizes thymus mass and morphological structure, decreases the number of necrotic damages and severity of dystrophic ones in healthy tissues, enhances anti-tumour effects of cytostatic chemotherapy. Herewith, betamide stimulates regenerative processes in normal cells. It activates endocytosis and transcytosis, enhances synthetic and metabolic processes, and in tumours betamide causes the induction of apoptosis and autophagocytosis.

Currently, preclinical research on betamide has been completely performed (the investigation of acute, subchronic, and chronic toxicities, specific activity, teratogenic, embryotoxic, and mutagenic effects). The technology for synthesis of the compound betamide has been developed and pilot batches have been obtained.

CONCLUSION

Due to the fact that plants are sources of numerous structurally different compounds with various compelling mechanisms of action and promising types of biological activity, the former play a fundamental role to play in a continuous process of a search for efficient and safe basic structures, i.e. synthetic platforms. The current methods and approaches towards the development of new drugs are based on inhibition or activation of enzymes and receptors. Plant metabolites, as an integral part of the living system, are not only compatible with its components but also have the specific binding ability with the most important cell systems, such as, for instance, its membrane, genic apparatus, or any of numerous proteins or polypeptides. The task of molecular design, in this case, is to produce a set of analogues based on this structural platform and, ultimately, to find a structure with the optimum combination of the required pharmacochemical characteristics.

The implementation of molecular design opportunities would require the further enhancement of works for the total synthesis of pharmacologically promising compounds, and therefore, would enhance the development of new synthetic methods (primarily environmentally friendly). These techniques should ensure an opportunity to efficiently obtain both target structures and a set of their analogues. This approach refers to high-tech organic synthesis with its enormous potential of the use towards the development of innovative new drugs. Further progress in the development of effective drugs would increasingly be determined by the indicated modern approach.

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