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Development of a Multistage Technology for the Industrial Synthesis of the Levosimendan API and Enantiomeric Separation of Intermediates

Abstract

A method for obtaining Levosimendan suitable for industrial application has been developed. Two literature routes for the synthesis have been evaluated. It has been found that the use of enantiopure (*R*)-2-chloropropionyl chloride in the initial step is ineffective due to racemization at the stage of the synthesis based on the malonic ester. Instead, a reported method based on the synthesis of the Levosimendan precursor, 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**), from racemic 2-bromopropionyl bromide has been modified to allow for scale-up and adaptation to industrial processes. A practical resolution method has been developed to isolate the (*R*)-enantiomer of amine **1** from the racemic mixture with a high enantiomeric purity (the content of (*R*)-enantiomer is up to 99 %). It has been shown that (*R*)-**1** can be converted to Levosimendan in a high yield without the stereochemical purity loss at the chiral center.

Keywords: Levosimendan; 4,5-dihydropyridazine-3(2*H*)-one; enantiomers separation; diastereomeric salts; *S*,*S*-2,3-*bis*-(4-methoxybenzoiloxy)butanedioic acid

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Розробка багатостадійної технології промислового синтезу АФІ левосимендану та енантіомерного розділення проміжних продуктів

Анотація

Розроблено метод одержання левосимендану, придатний для промислового використання. Перевірено два літературних методи синтезу левосимендану та з'ясовано, що використання (*R*)-2-хлоропропіонілхлориду на першій стадії не ефективне через рацемізацію на стадії синтезу на основі малонового естеру. Літературний метод синтезу попередника левосимендану, 6-(4-амінофеніл)-5-метил-4,5-дигідропіридазин-3(2*H*)-ону (**1**), було модифіковано з використанням рацемічного 2-бромопропіонілброміду з метою зробити його придатним для масштабування та перетворення на промислову технологію. Розроблено метод виділення (*R*)-енантіомеру аміну **1** з рацемату з високою енантіомерною чистотою (вміст (*R*)-енантіомеру до 99 %). Показано, що амін (*R*)-**1** може бути перетворено на левосимендан зі збереженням конфігурації хірального центру та високим виходом.

Ключові слова: левосимендан; 4,5-дигідропіридазин-3(2*H*)-он; розділення енантіомерів; діастереомерні солі; *S*,*S*-2,3-*біс*-(4-метоксибензоїлокси)бутандіова кислота

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■ Introduction

Levosimendan (**Figure 1**) represents a new class of cardiotonic agents reported as Ca^{2+} sensitizers without cardiovascular side effects. It increases the sensitivity of contractile proteins to calcium by binding to cardiotroponin C. Levosimendan increases the strength of contractions but does not depend on the ventricular relaxation. In addition, Levosimendan opens ATP-sensitive potassium channels in the vascular smooth muscle, stimulating the vasodilation of systemic arteries, coronary arteries, and systemic veins. It is a selective phosphodiesterase III inhibitor *in vitro*. In patients with heart failure, the positive inotropic and vasodilator effects of Levosimendan lead to an increase in the contractile force of the myocardium and a decrease in the preload and postload without the adverse impact on the diastolic function. Levosimendan activates the "ischemic" myocardium in patients after coronary angioplasty or thrombolysis [1–3]. Levosimendan is used for the short-term treatment of acute, severe decompensated chronic heart failure [2]. This drug is registered in Ukraine, and at least one domestic manufacturer, JSC Farmak, is present in the pharmaceutical market of Ukraine.

Due to the complicated logistics, the development of a domestic industrial method for the synthesis of Levosimendan is an urgent task. The commercial production of Levosimendan requires a cheap, convenient, and efficient synthetic approach, which can be converted into technology.

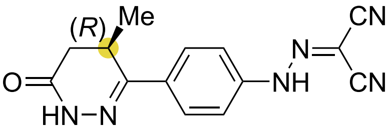


Figure 1. The structure of Levosimendan

In all methods of the synthesis of Levosimendan described, the last stage is common, namely the conversion of the *R*-enantiomer of amine **1** ((*R*)**-1**) into a diazo compound, followed by the interaction with malononitrile, as it has been presented in the patent [4] (**Scheme 1**). This makes amine **1** the key compound in the synthesis of Levosimendan.

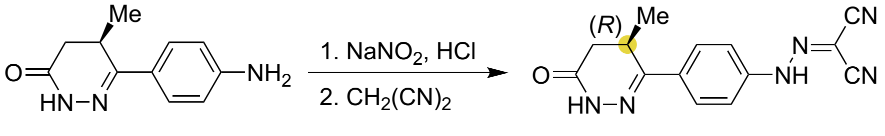
The scientific literature describes the four main approaches to 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one **1** [5–9] (Routes 1–4, **Schemes 2–5**).

Scheme 2 (Route 1) shows the method based on utilizing the chiral starting materials from the first stage [5]. The authors reported that this route led to a crude amine (*R*)**-1** with 84% *ee* in the total yield of 30%, and further recrystallization of the crude product from ethyl acetate increased *ee* up to 98%.

Similar methods of the preparation of amine **1** starting from racemic 2-bromopropionyl bromide [6] or propionyl chloride [7] were described with the total yields of racemic compound **1** of 58% and 22%, respectively (Route 2, **Scheme 3**). In the work by *Wang et al.* [7], the separation of pyridazine-3(2*H*)-one **1** enantiomers *via* the crystallization of diastereomeric salts with *L*-(+)-tartaric acid was reported with the yield of 8%.

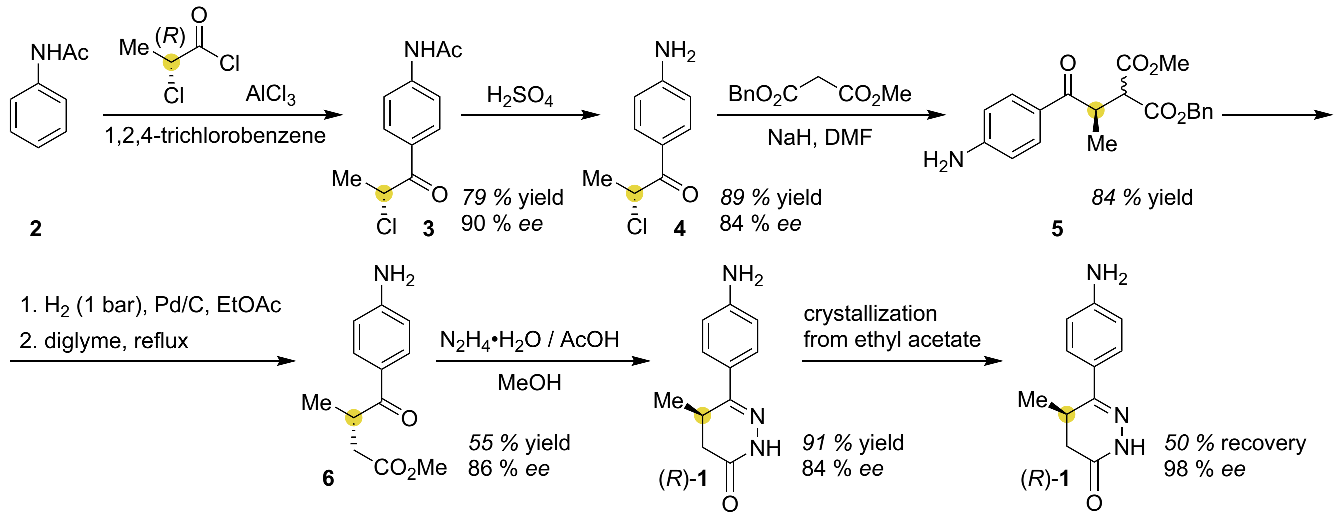
If a racemic 2-chloropropionyl chloride was used in such a route, racemic amine **1** was obtained with the total yield of 23% [8].

The preparation of the enantiomerically pure amine (*R*)**-1** starting from acetanilide **2** and citraconic anhydride (Route 3, **Scheme 4**) was described in [9]. This method includes a double bond reduction stage by hydrogen in the presence of a chiral Ru-catalyst (*S*)-BINAP-Ru. The total yield of the product (*R*)**-1** was ~14%.

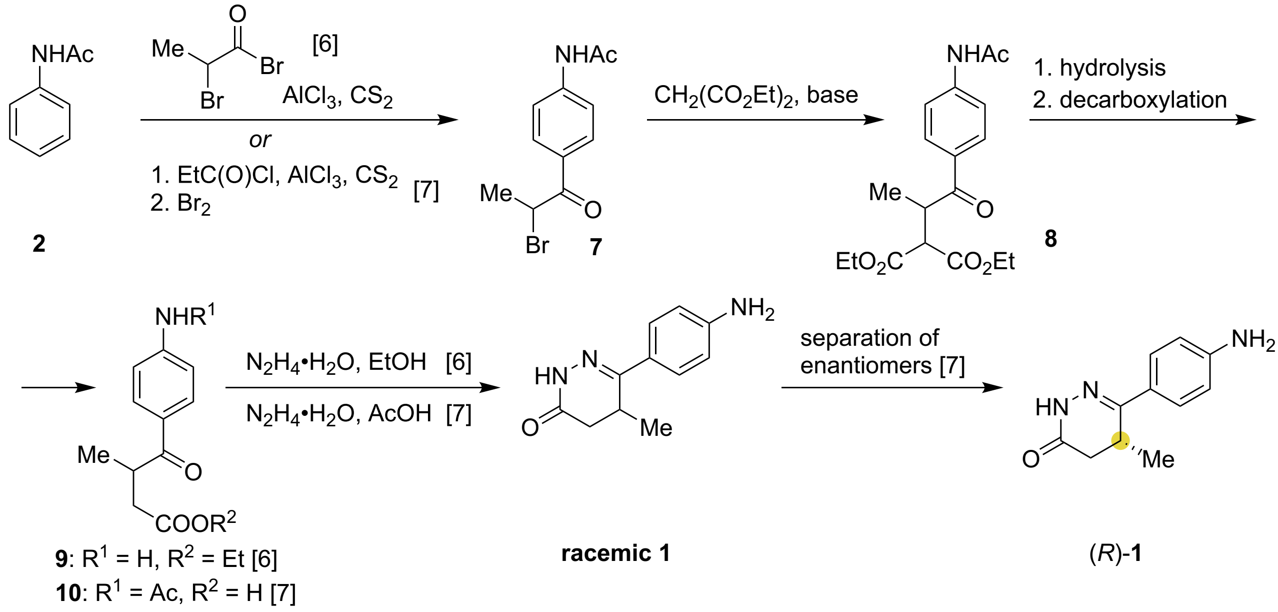


(*R*)**-1** **Levosimendan**, *80 %*

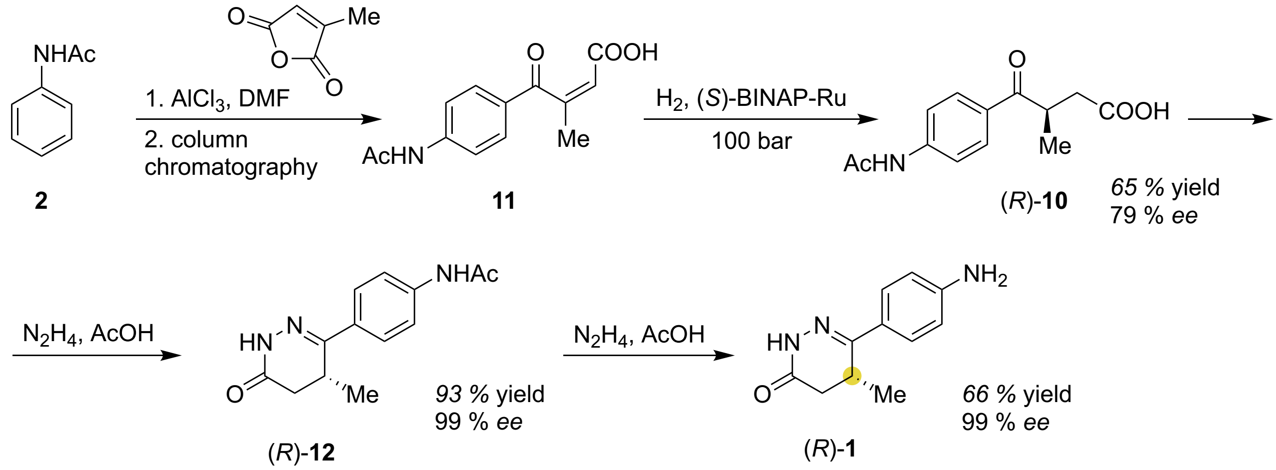
Scheme 1. The last stage of the Levosimendan synthesis [4]



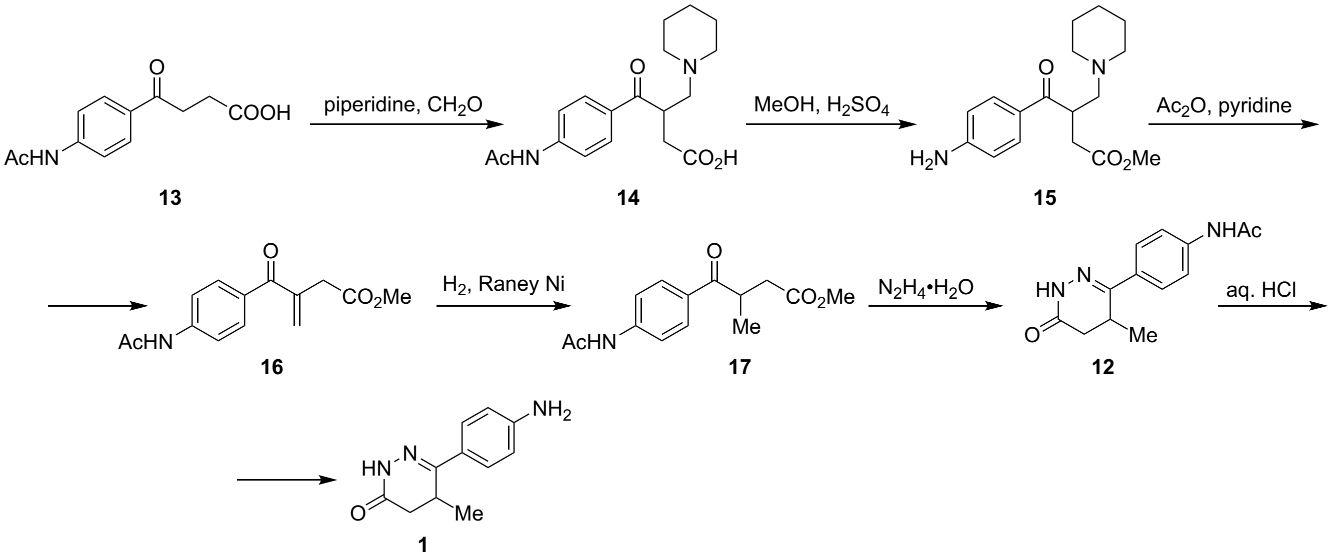
Scheme 2. The synthesis of 6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one starting from (*R*)-2-chloropropionyl chloride [5]



Scheme 3. The preparation of 6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one starting from racemic 2-bromopropionyl bromide or propionyl chloride [6, 7]



Scheme 4. The preparation of the enantiomerically pure (*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one starting from citraconic anhydride *via* the double bond reduction stage [9]



Scheme 5. The preparation of racemic 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one using 4-(4-acetamidophenyl)-4-oxobutanoic acid [8]

Another method for the preparation of racemic amine **1** using keto-acid **13** with the total yield of 28% was reported in [8] (Route 4, **Scheme 5**). This method, like the previous one, includes a double bond reduction with hydrogen on a Raney Ni catalyst.

As can be seen from the literature data, the enantiomerically pure amine (*R*)**-1** was obtained either by using chiral substances from the first stage [5], or by creating the desired configuration of the chiral center during the synthesis process [9], or by separating the racemate of amine **1** [4, 7]. The comparison of the aforementioned synthetic pathways is shown in **Table 1**.

The aim of this study was to evaluate the known methods of synthesis of Levosimendan and develop an optimal synthetic route suitable for converting into the technology and production of Levosimendan on the industrial scale.

■ Results and discussion

The synthesis pathways to 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**), including the stage of reduction with gaseous hydrogen (Routes 3, 4; **Schemes 4**, **5**), are unsuitable for industrial use at JSC “Farmak” due to the lack of specific conditions for working with gaseous hydrogen under high pressure.

Therefore, for our research, we chose two methods shown in **Scheme 2** [5] and **Scheme 3** [6] (Routes 1, 2), starting from (*R*)-2-chloropropionyl chloride or 2-bromopropionyl bromide, respectively.

Table 1. The comparison of the literature synthetic pathways of 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one

Route 1 (**Scheme 2** [5])	**Route 2** (**Scheme 3** [6, 7])	**Route 3** (**Scheme 4** [9])	**Route 4** (**Scheme 5** [8])
• total yield – 30 % • *ee* – 84 %	• total yield – 22-58 %[a] • racemate	• total yield – 14 % • *ee* – 99 %	• total yield – 28 % • racemate
+ high *ee*[b] + without the enantiomer separation	+ cheap and available starting materials	+ creation of a chiral center with high *ee* in the synthesis process + without the enantiomer separation	+ cheap and available starting materials
– the use of a non-commercial unsymmetrical malonic ester[c] – the additional stage of the benzyl methyl malonate synthesis	– the low total yield starting from 2-propionyl chloride [7] – the low yield (8 %) at the enantiomer separation stage [7]	– the mixture of products at the first stage requires the column chromatography for separation – the low yield (34 %) of desired product at the first stage – the use of **gaseous hydrogen** – expensive Ru-catalysts – the low total yield	– the use of **gaseous hydrogen** – expensive catalysts

Notes: [a] 22 % yield starting from propionyl chloride [7]; 58 % yield starting from 2-bromopropionyl bromide [6]; [b] “+” indicates advantages of the method; [c] “–” indicates disadvantages of the method

The authors in [5] (**Scheme 2**) noted that the use of unsymmetrical benzyl methyl malonate allowed obtaining compound **6** under mild conditions while preserving the configuration of the chiral center. In addition, it was stated that the use of aniline **4** (rather than acyl-protected aniline **3**) in the reaction with malonic ester allowed them to analyze the enantiomeric purity of the product by the HPLC method using chromatographic columns with a chiral stationary phase.

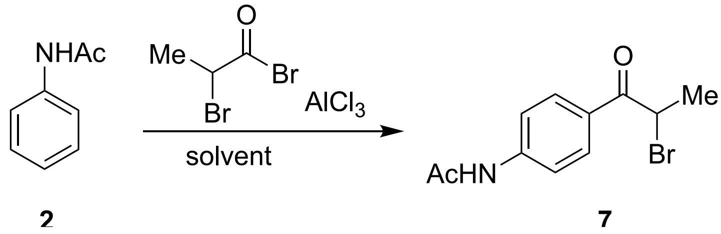
While reproducing this scheme (**Scheme 2**), we faced some difficulties. Compound **4** was obtained as described in [5] with the same yield and *ee*. However, the following stages gave unexpected and disappointing results. The interaction of chloride **4** with unsymmetrical malonic ester using the conditions described proceeded very slowly. To achieve a conversion of 90%, it was necessary to carry out the reaction for 6 days, contrary to 24 h in [5]. Moreover, the analysis of the enantiomeric purity of product **5** showed that during the interaction of compound **4** with benzyl methyl malonate, the racemization of the initial chiral center occurred. In addition, unreacted amine **4** with the initial *ee* of 92% was recovered from the reaction mixture as a racemate. This indicates that the racemization occurred in starting amine **4** due to the enolization of the keto function under the action of the base. Attempts to use a symmetrical dimethyl malonate in the reaction with either amine **4** or acylated amine **3** resulted in the racemization of the initial chiral center as well.

This failure showed that the utilization of a chiral starting material, namely (*R*)-2-chloropropionyl chloride, was ineffective. Therefore, we evaluated the second selected route starting from 2-bromopropionyl bromide [6] (**Scheme 3**) with the following separation of the enantiomers of amine **1** *via* the crystallization of diastereomeric salts.

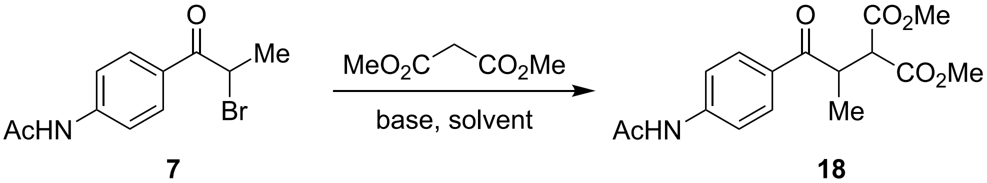
The first stage of the synthesis, namely the Friedel-Crafts reaction, was described in [6] using carbon disulfide as a solvent. This compound is very toxic and flammable; therefore, it is unsuitable for application in modern pharmaceutical manufacturing. We tested other organic solvents in this reaction to find the optimal conditions that would give high yields of product **7** (**Scheme 6**). Thus, heating the reaction mixture in dichloroethane at 80°C for 1.5 h led to the significant tar formation, and as a result, the product isolation failed. If the reaction mixture was refluxed in dichloromethane for 4 or 6 h, the conversion of acetanilide **2** was about 90%. An isolated yield of product **7** was 80% after column chromatography. The best results were achieved in 1,2,4-trichlorobenzene at 80°C after heating for 3 h, as described for (*R*)-2-chloropropionyl chloride in [5]. Product **7** was isolated in the yield of 99%.

The malonic synthesis was described using diethyl malonate, sodium hydride as a base and THF as a solvent [6]. We used dimethylmalonate instead of diethylmalonate in our research (**Scheme 7**). This choice is made as at the next step of hydrolysis/decarboxylation, an alcoholic solution of HCl is used, and the solution of HCl in methanol is a commercial reagent. In addition, it is difficult to obtain such a solution from ethanol in the laboratory or industry since absolute alcohol is required to prepare the solution.

Sodium hydride or potassium *tert*-butoxide was used as a base, and DMF or THF as a solvent. **Table 2** summarizes the results of the experiments.



Scheme 6. The Friedel-Crafts reaction of acetanilide and 2-bromopropionyl bromide



Scheme 7. The synthesis of dimethyl 2-(1-(4-acetamidophenyl)-1-oxopropan-2-yl)malonate via the malonic ester synthesis

Table 2. The malonic ester synthesis – optimization of reaction conditions

Entry	Base	Equiv. of the base and dimethyl malonate	Solvent	Isolated yield of **18**, %	LC-MS purity of **18**, %
1	NaH	2	DMF	30	78
2	*t*BuOK	2	DMF	67	83
3	*t*BuOK	2	THF	97	100
4	*t*BuOK	1	THF	94	93
5	*t*BuOK	1.25	THF	96	93

It is evident from **Table 2** that *t*BuOK gave better results if compared with sodium hydride, and THF was a better solvent than DMF for this reaction. It should be noted that when using 1.0 equiv. *t*BuOK and dimethyl malonate in THF the starting bromide in the amount of up to 1% was identified in the ^{1}H NMR spectrum. The use of 1.25 equiv. *t*BuOK and dimethyl malonate in THF was shown to be optimal (see *entry 5*).

It is noteworthy to mention that attempts to purify product **18** by the recrystallization were unsuccessful. However, it was found that all the impurities present in the product after the malonic synthesis disappeared after the next stage of the hydrolysis/decarboxylation sequence. Therefore, we believe that it is impractical to purify compound **18** before the next step.

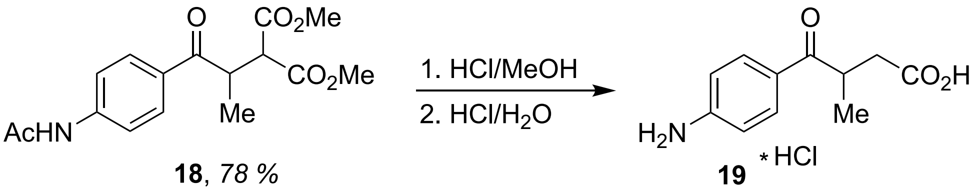
The stages of hydrolysis and decarboxylation (**Scheme** 8) were carried out similarly to the transformations described in [6]. As we used dimethyl ester instead of diethyl, we also used the methanol solution rather than the ethanol solution of HCl.

The cyclization reaction (**Scheme 9**) was performed according to [6]. We only slightly modified amine **1** isolation since the procedure described was not reproducible. If the precipitate product was isolated by filtration from the ethanolic reaction mixture as described in [6], the yield of amine **1** was up to 50%. Therefore, we first removed the solvent (EtOH) from the reaction mixture to dryness and then treated the residue with water. The precipitate formed was filtered and purified as described in the article [6], yielding pure amine **1** with a high yield.

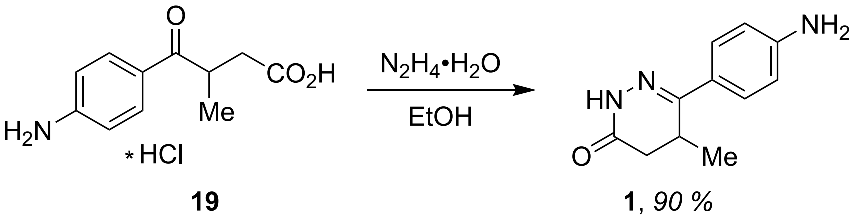
Thus, the overall optimized scheme for the synthesis of racemic amine **1** is as follows (**Scheme 10**) with the total isolated yield of 67%. All stages can be easily scaled up in the laboratory and amount of each substance obtained per loading is shown in **Scheme 10**.

The separation of racemic amine **1** was described in [7] and included the following stages: **(1)** the synthesis of the salt with *L*-(+)-tartaric acid; **(2)** the crystallization of the resulting mixture of diastereomeric salts using 2-propanol; **(3)** the conversion of the salt to amine **1**. It was mentioned in the article that one crystallization was required for the complete separation of enantiomers.

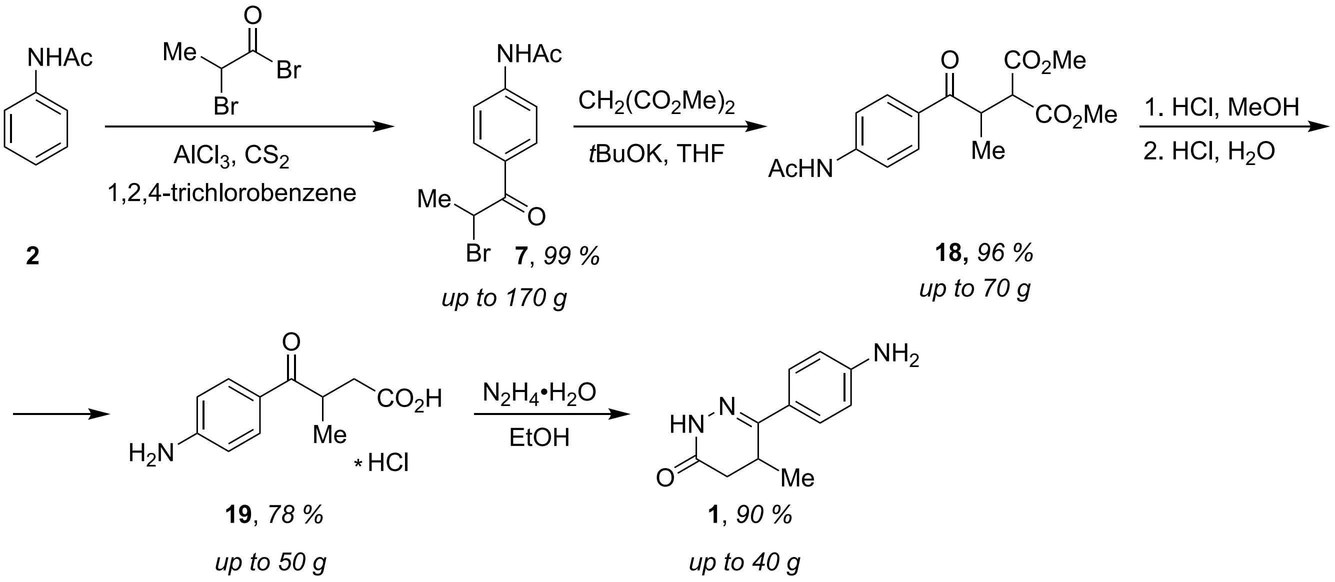
We have found that amine **1** with (*R*)-enantiomer content >98% can be reached by 20–32 crystallizations of diastereomeric salts, depending on the amount of the starting mixture of diastereomeric salts. If 4.5 g of the mixture was used for the separation, the (*R*)-enantiomer content of 98.4% was reached after 20 crystallizations. At the same time, 45 g of the mixture of diastereomeric salts needed 32 crystallizations



Scheme 8. The preparation of 4-(4-Aminophenyl)-3-methyl-4-oxobutanoic acid hydrochloride *via* the hydrolysis and decarboxylation of dimethyl 2-(1-(4-acetamidophenyl)-1-oxopropan-2-yl)malonate



Scheme 9. The cyclization reaction of 4-(4-aminophenyl)-3-methyl-4-oxobutanoic acid hydrochloride with hydrazine hydrate



Scheme 10. The optimized scheme for the synthesis of racemic 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one

for the isolation of the *R*-enantiomer with its content of 98.6%.

The application of tartaric acid derivatives **20**,**21** for the separation of enantiomers of 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**) for the further use of the desired *R*-enantiomer in the synthesis of Levosimendan was described in the patent [4]. The patent stated that any derivative **20**,**21** with any configuration of chiral centers can be used for the separation (**Figure 2**). The separation of enantiomers using acids **20**,**21** included the following stages as in the case of *L*-(+)-tartaric acid: **(1)** the synthesis of the salt with tartaric acid derivative **20**,**21**; **(2)** the crystallization of the resulting mixture of diastereomeric salts using the ethanol/H_2O 1:1 mixture; **(3)** the conversion of the salt to amine **1**. Authors of [4] stated that one crystallization was sufficient to separate diastereomeric salts. Depending on the configuration of acids **20**,**21** used, the desired (*R*)-**1** either precipitated or remained in the solution.

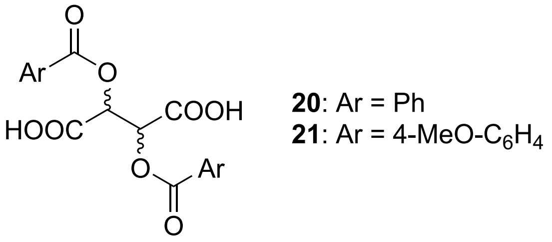
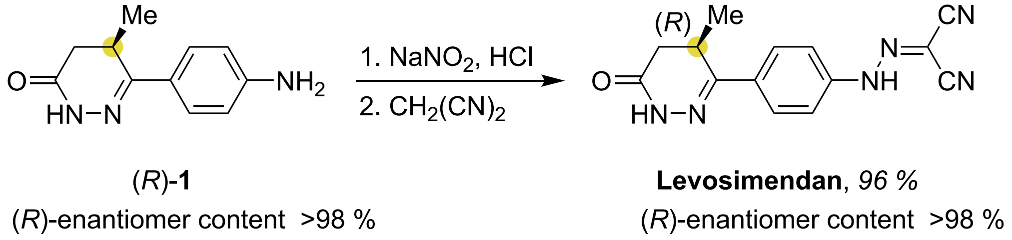


Figure 2. Tartaric acid derivatives used for the separation of enantiomers of amines [4]

When we used acid **20** for the enantiomer separation as it was described in patent [4], after two crystallizations of diastereomeric salts we obtained racemic amine **1** without any enrichment with a desired (*R*)-enantiomer. We showed that the best results were achieved when using a small excess of *S*,*S*-2,3-*bis*-(4-methoxybenzoyloxy)butanedioic acid (**21**). One crystallization of the mixture of diastereomeric salts of racemic amine **1**, as it was described in [4], did not lead to the full enantiomer separation. The *R*-enantiomer of amine **1** with the (*R*)-enantiomer content of up to 99% was isolated after five crystallizations of the mixture of diastereomeric salts.

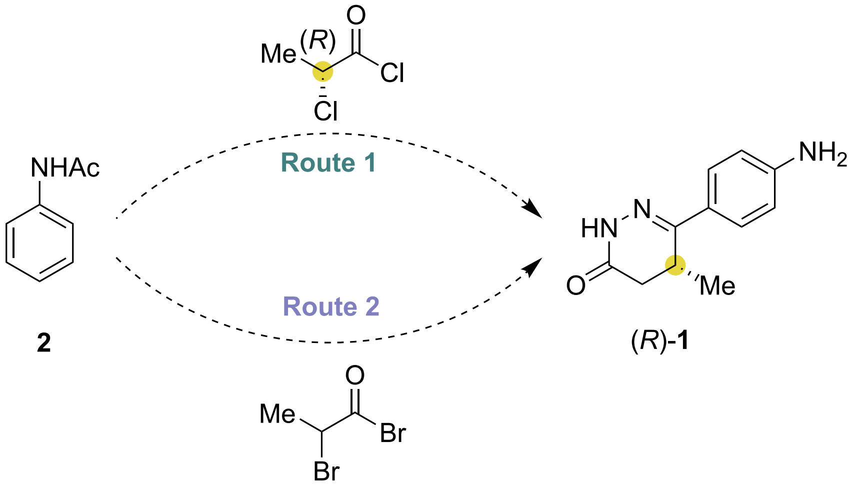
Table 3 summarizes our results of both methods selected for obtaining (*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**).

(*R*)-**1** was easily converted into Levosimendan as it was described in [4] with preserving the configuration of the chiral center and the yield of 96% (**Scheme 11**).



Scheme 11. The last step for the preparation of Levosimendan

Table 3. A brief summary of our results for obtaining (*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**)



Route 1	Route 2
– the expensive chiral starting compound[a] – racemization on the malonic synthesis stage	+ high yields at all stages[b] + 67 % total yield of the racemate + 24 % yield at the enantiomer separation stage + the separation of enantiomers up to 99 % of the (*R*)-enantiomer content + scalable

Notes: [a] "–" indicates disadvantages of the method; [b] "+" indicates advantages of the method

The (*R*)-Enantiomer content was the main quality indicator that was focused on in the process of the synthetic route optimization.

Since Levosimendan is the active pharmaceutical ingredient (API), it is important to check certain quality parameters specific to such substances. The quality tests were performed according to the following general chapters of the European Pharmacopoeia (EP): identification by NMR (2.2.33), loss on drying (2.2.32), sulfated ash (2.4.14), and HPLC assay, related substances, and (*R*)-enantiomer content tests (2.2.29, 2.2.46) [12]. The results of the final sample quality of the Levosimendan obtained are shown in **Table 4**.

Table 4. The results of quality testing of the final sample of Levosimendan

Parameter	Eur. Ph. General Chapter	Results
Appearance		A dark-yellow crystalline powder
Solubility		Very slightly soluble in ethanol (96 %)
Identification	2.2.33	Conform to the structure
pH	2.2.3	6.00
Loss on drying	2.2.32	1.26 %
Sulfated ash	2.4.14	3.13 %
Impurities: • individual impurity • total impurities	2.2.29, 2.2.46	0.30 % 1.21 %
(*R*)-Enantiomer content		up to 99 %
Assay		93.1 %

■ Conclusions

We have evaluated two literature methods for obtaining 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**). It has been found that the use of (*R*)-2-chloropropionyl chloride in the initial step is ineffective due to racemization at the stage of the synthesis based on the malonic ester. The method of the synthesis of 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**1**) starting from racemic 2-bromopropionyl bromide has been modified to be suitable for scaling up and used for converting it into the industrial technology. The separation of racemic 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one yielding *R*-enantiomer with the high enantiomeric purity ((*R*)-enantiomer content of up to 99%) has been developed. It has been shown that amine (*R*)-**1** can be converted into Levosimendan with the configuration retention of the chiral center and a high yield.

Experimental part

^{1}H NMR spectra were recorded using a Varian VXR-300 instrument at 300 MHz, or a Varian UNITY-Plus 400 instrument at 400 MHz. ^{13}C NMR spectra (proton decoupled) were recorded on a Bruker AVANCE DRX 500 instrument at 125 MHz. The chemical shifts are given in ppm relative to TMS as an internal or external standard.

Thin-layer chromatography (TLC) was carried out on aluminium-backed plates coated with silica gel (Merck Kieselgel 60 F254). LC-MS spectra were registered on an "Agilent 1100 Series" instrument with a diode-matrix and an "Agilent 1100 LS/MSD SL" mass-selective detector (the ionization method – chemical ionization at atmospheric pressure; the ionization chamber operation conditions – simultaneous scanning of positive and negative ions in the range of 80–1000 m/z).

Melting points were determined in open capillaries using a SMP3 instrument (Stuart Scientific Bibby Sterlin Ltd, Stone, Staffordshire, UK).

Unless otherwise stated, commercially available reagents were used without purification after being purchased. The solvents were purified according to standard procedures.

***N*-(4-(2-Bromopropanoyl)phenyl)acetamide (7)**

Acetanilide **2** (30 g, 0.222 mol) in 1,2,4-trichlorobenzene (500 mL) was placed into a three-necked flask equipped with a thermometer, a mechanical stirrer, and an addition funnel. Anhydrous aluminum chloride (90 g, 0.667 mol) was added to the solution. The reaction mixture was heated to 70°C, and 2-bromopropionyl bromide (60 g, 0.25 mol) was added dropwise for ~30 min at such a rate that the temperature did not rise higher than 80°C. After the addition was completed, the reaction mixture was heated at 80°C for 3 h. The heating source was removed, and dichloromethane (500 mL) was added to the reaction mixture at 80°C. The temperature of the reaction mixture decreased to (50±5)°C. The warm reaction mixture was slowly poured into the ice/water mixture (1 L). The organic layer was separated, and the product was extracted from water with dichloromethane (5×100 mL). Dichloromethane solutions were combined, washed with water (5×70 mL), and dried over $MgSO_4$. The solvent was evaporated in vacuum. The residue containing product **7** and 1,2,4-trichlorobenzene was washed with hexane (5×200 mL) to remove 1,2,4-trichlorobenzene. After removing 1,2,4-trichlorobenzene, the residue was placed into the flask and dried in vacuum (0.5–1 mm Hg) at 50°C for 4–5 h.

A yellow solid. Yield – 59 g (99%). M. p. 118–120°C (Lit. 128–131°C [6]). ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 1.88 (3H, d, $^3J_{HH}$ = 6.6 Hz, CH_3), 2.21 (3H, s, $COCH_3$), 5.26 (1H, q, $^3J_{HH}$ = 6.6 Hz, CH), 7.65 (2H, d, $^3J_{HH}$ = 8.7 Hz, ArH), 7.98 (2H, d, $^3J_{HH}$ = 8.7 Hz, ArH). (Lit. [10])

Dimethyl 2-(1-(4-acetamidophenyl)-1-oxopropan-2-yl)malonate (18)

Dimethyl malonate (37 g, 0.28 mol) was added dropwise to the solution of *t*BuOK (31 g, 0.28 mol) in THF (350 mL). The reaction mixture was stirred at room temperature (15–20°C) for 1 h, and bromide **7** (60 g, 0.22 mol) in THF (300 mL) was slowly added at the same temperature. The reaction mixture was stirred at room temperature (15–20°C) for 48 h (until the disappearance of the starting bromide **7** by TLC, eluent hexane/MTBE 1:5, R_f (**7**) = 0.3, R_f (**18**) = 0.2), and then poured into water (700 mL). The pH of the solution was adjusted to 5.5–6 using 10% HCl solution. The product was extracted with MTBE (4×300 mL), and the organic layer was washed with water (3×250 mL) and dried over $MgSO_4$. The solvent was distilled off in vacuum (10 mm Hg), then the residual solvent and the excess of dimethyl malonate were removed in vacuum (1 mm Hg, 60°C, 6 h), yielding **18** as a yellow solid (68 g, 96%).

^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 1.18 (3H, d, $^3J_{HH}$ = 7.2 Hz, CH_3), 2.20 (3H, s, $COCH_3$), 3.66 (3H, s, $COOCH_3$), 3.80 (3H, s, $COOCH_3$), 4.00 (1H, d, $^3J_{HH}$ = 10.9 Hz, CH), 4.12–4.14 (1H, m, CH), 7.60 (2H, d, $^3J_{HH}$ = 8.1 Hz, ArH), 7.95 (2H, d, $^3J_{HH}$ = 8.1 Hz, ArH). LC-MS, m/z: 320 $[M–H]^-$ (negative ionization); 322 $[M+H]^+$ (positive ionization).

4-(4-Aminophenyl)-3-methyl-4-oxobutanoic acid hydrochloride (19)

To compound **18** (74 g, 0.23 mol), the solution of HCl in methanol (1.4 L, c = 3 mol L^{-1}) was added, and the reaction mixture was refluxed for 8 h. The solvent was removed to dryness in vacuum, and an aqueous solution of HCl (1.4 L, c = 6 mol L^{-1}) was added to the residue. The resulting solution was refluxed for 5 h. The solvent was evaporated to dryness in vacuum, and 50 mL of conc. HCl was added to the residue. The precipitate was filtered off and washed with conc. HCl (10 mL) and 2-propanol (40 mL), and dried in vacuum (1 mm Hg, 60°C, 4 h).

A brownish solid. Yield – 43.5 g (78%). M. p. 186–188°C (dec.) (Lit. 187–188°C (dec.) [6]). ^{1}H NMR (300 MHz, D_2O), δ, ppm: 1.16 (3H, d, $^3J_{HH}$ = 7.2 Hz,

CH_3), 2.62 (1H, dd, $^2J_{HH}$ = 17.2 Hz, $^3J_{HH}$ = 4.8 Hz, CH_2), 2.85 (1H, dd, $^2J_{HH}$ = 17.2 Hz, $^3J_{HH}$ = 9.3 Hz, CH_2), 3.91-3.99 (1H, m, CH), 7.44 (2H, d, $^3J_{HH}$ = 8.7 Hz, ArH), 8.07 (2H, d, $^3J_{HH}$ = 8.7 Hz, ArH).

6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (1)

The hydrazine hydrate solution (90 mL, 90% aqueous solution) was added to the solution of compound **19** (44 g, 0.18 mol) in ethanol (500 mL). The reaction mixture was refluxed for 5 h and then cooled to room temperature. The solvent was removed in vacuum to dryness. Water (50 mL) was added to the residue. The precipitate was filtered off, washed with water and 2-propanol, and dried in vacuum (1 mm Hg, 50 °C, 4 h).

A brownish solid. Yield – 33 g (90%). M. p. 193–195°C (Lit. 211–212°C [9], 195–197°C [6]). ^{1}H NMR (300 MHz, DMSO-d_6), δ, ppm: 1.03 (3H, d, $^3J_{HH}$ = 7.2 Hz, CH_3), 2.17 (1H, d, $^2J_{HH}$ = 16.7 Hz, CH_2), 2.59 (1H, dd, $^2J_{HH}$ = 16.7 Hz, $^3J_{HH}$ = 6.6 Hz, CH_2), 3.23–3.29 (1H, m, CH), 5.49 (2H, s, NH_2), 6.57 (2H, d, $^3J_{HH}$ = 8.4 Hz, ArH), 7.48 (2H, d, $^3J_{HH}$ = 8.4 Hz, ArH), 10.67 (1H, s, NH). (Lit. [5])

(*R*)-6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one ((*R*)-1). The enantiomer separation using *L*-(+)-tartaric acid

The racemate of amine **1** (23 g, 0.11 mol), *L*-(+)-tartaric acid (34 g, 0.23 mol), and 2-propanol (450 mL) were placed into a flask and heated under reflux for 30 min. The reaction mixture was allowed to cool to room temperature overnight. The precipitate was filtered off and dried in vacuum (1 mm Hg, 40°C, 5 h), yielding 45 g (100%) of a mixture of diastereomeric salts as a solvate with one molecule of 2-propanol.

The mixture of diastereomeric salts was crystallized from 2-propanol 32 times (until the (*R*)-enantiomer content in the sample reached 98.6%). The first crystallization was carried out using activated carbon. As the amount of the precipitate used for the crystallization decreased, the amount of the solvent decreased proportionally (for example, 450 mL of the solvent was used for 45 g of the precipitate, and 300 mL of the solvent was used for 30 g of the precipitate).

When required the (*R*)-enantiomer content in the sample was reached, (*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one *L*-(+)-tartrate was converted to (*R*)-6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one ((*R*)-**1**) as follows.

An aqueous solution of potassium carbonate was added to the suspension of (*R*)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one *L*-(+)-tartrate (7 g, 21 mmol) in water (50 mL) to pH 8. The precipitate was filtered off, washed with the aqueous solution of potassium carbonate (15 mL) and water (2×35 mL), and dried in vacuum (1 mm Hg, 40°C, 5 hours), yielding 3 g (86%) of amine (*R*)-**1** with the (*R*)-enantiomer content of 98.6%.

(*R*)-6-(4-Aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one ((*R*)-1). Enantiomers separation using *S*,*S*-2,3-*bis*-(4-methoxybenzoyloxy)butanedioic acid (21)

The racemate of amine **1** (23 g, 0.11 mol) was placed into a 1-L flask, ethanol (200 mL) was added, and the suspension formed was heated at 65°C for 30 min, followed by the addition of *S*,*S*-2,3-*bis*-(4-methoxybenzoyloxy)butanedioic acid (**21**) (57 g, 0.136 mol) and water (200 mL) at the same temperature. The resulting solution was heated at 65°C for 1 h, and then refluxed for 5 min. The reaction mixture was allowed to cool to room temperature. In 24 h, the precipitate was filtered off and used for further crystallization.

After obtaining the salt or after the preliminary crystallization, the crude precipitate was placed into a flask, the solvent (ethanol/water 1:1) was added, and the resulting solution was refluxed for 5–10 min and allowed to slowly cool to room temperature. In 24 h, the precipitate was filtered and used for the subsequent crystallization. For the first crystallization, approximately the same amount of the solvent was used as for the preparation of the salt. During the subsequent crystallizations, the amount of the solvent was gradually reduced (1/3 of the previous volume of the solvent was added, the suspension was heated to boiling, and more solvent was added in portions until a clear solution was formed). The first crystallization was carried out using activated carbon. After the fifth crystallization, the precipitate was dried in vacuum (1 mm Hg, 65°C, 1 h) from alcohol residues and used for conversion to the amine.

A 25% aqueous solution of ammonia was added to the suspension of the salt of amine ((*R*)-**1**) with acid (**21**) in water (~4 mL of water per 1 g of the salt) to pH 8. The suspension was stirred for 15–20 min, and the pH was again controlled. If the medium became neutral, the solution of ammonia was added to pH 8. The addition of the ammonia solution was repeated until the pH of the medium (pH 8) did not change while stirring for 30–40 min. The precipitate was filtered off, washed with water, and dried in vacuum (1 mm Hg, 60°C, 2 h), yielding 2.8 g (12% based on the racemate, 24% based on the content of the *R*-enantiomer in the racemate) of amine ((*R*)-**1**) with the (*R*)-enantiomer content of up to 99%.

(*R*)-N-(4-(4-Methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl)-carbono-hydrazonoyl dicyanide (Levosimendan)

The solution of sodium nitrite (1.16 g, 17 mmol) in water (20 mL) was added dropwise to the solution of amine (*R*)-**1** (3.4 g, 17 mmol, the (*R*)-enantiomer content >98%) in a diluted HCl (prepared from 10 mL of conc. HCl and 135 mL of water) at 10–12°C. The reaction mixture was stirred at this temperature for 10 min, and the solution of malononitrile (1.11 g, 17 mmol) in water (20 mL) was added dropwise at the same temperature. The reaction mixture was stirred at room temperature (20°C) for 1 h and then neutralized with an aqueous solution of sodium acetate to pH 7. The suspension formed was stirred for 30 min at room temperature. The precipitate was filtered off, washed with water (4×50 mL), and dried in vacuum (1 mm Hg, 65°C, 4 h).

A yellow solid. Yield – 4.5 g (96%). M. p. >210°C (dec) (Lit. 210–214°C [13]). (*R*)-enantiomer content >98%. ^{1}H NMR (400 MHz, DMSO-d$_6$), δ, ppm: 1.07 (3H, d, $^3J_{HH} = 6.4$ Hz, CH$_3$), 2.24 (1H, d, $^2J_{HH} = 16.4$ Hz, CH$_2$), 2.65 (1H, dd, $^2J_{HH} = 16.4$ Hz, $^3J_{HH} = 6.0$ Hz, CH$_2$), 3.33–3.37 (1H, m, CH), 7.48 (2H, d, $^3J_{HH} = 8.0$ Hz, ArH), 7.81 (2H, d, $^3J_{HH} = 8.0$ Hz, ArH), 10.96 (1H, s, NH), 12.77–13.32 (1H, br.s, NH). ^{13}C NMR (125.6 MHz, DMSO-d$_6$), δ, ppm: 16.8, 28.0, 34.4, 86.2, 110.7, 115.1, 117.4, 127.9, 133.0, 142.8, 152.7, 167.3. (Lit. [11]).

The (*R*)-enantiomer content analysis

The (*R*)-enantiomer content was determined by the HPLC method on an Agilent 1260 Infinity II instrument. The separation was performed on a Lux Cellulose-3 column (250 mm × 4.6 mm and 5 µm particle size) at a flow rate of 1.0 mL min^{-1} and the UV detection at 311 nm. The column temperature was set at 25°C. The isocratic elution was carried out using a mobile phase composed of water, acetonitrile, and acetic acid (60:40:0.1 *v*/*v*/*v*, respectively).

In a typical analysis system, the suitability solution containing Levosimendan and Dextrosimendan (0.25 mg mL^{-1}, Clearsynth) was used. It was previously dissolved in DMSO and then diluted with the mobile phase. The test solution was prepared in the same way at a concentration of about 1 mg mL^{-1}. The volume of the injected solutions was 10 µL.

Typical chromatograms of blank, system suitability, and test solutions are shown in **Figure 3**. The chromatographic system was considered suitable if the resolution between Levosimendan and Dextrosimendan peaks calculated from the system suitability solution chromatogram was not less than 2.0.

The Levosimendan assay in the final sample was carried out by the HPLC method on an Agilent 1260 Infinity II instrument. A Hypersil BDS C18 column (250 mm × 4.6 mm and 5 µm particle size) was used as the stationary phase. The column temperature was set at 40°C. A mobile phase mixture of acetonitrile, water, and 0.1% trifluoroacetic acid solution adjusted to pH 3.0 with triethylamine (40:10:50 *v*/*v*/*v*, respectively) was used. The isocratic elution was performed at a flow rate of 1.0 mL min^{-1} with the UV detection at 375 nm.

As a reference solution, 0.1 mg mL^{-1} Levosimendan solution, previously dissolved in DMSO and then diluted with mobile phase, was used. The test solution was prepared in the same way in the same concentration. The volume of the injected solutions was 10 µL.

Typical chromatograms of blank, reference, and test solutions are shown in **Figure 4**. The chromatographic system was considered suitable if the relative standard deviation calculated for

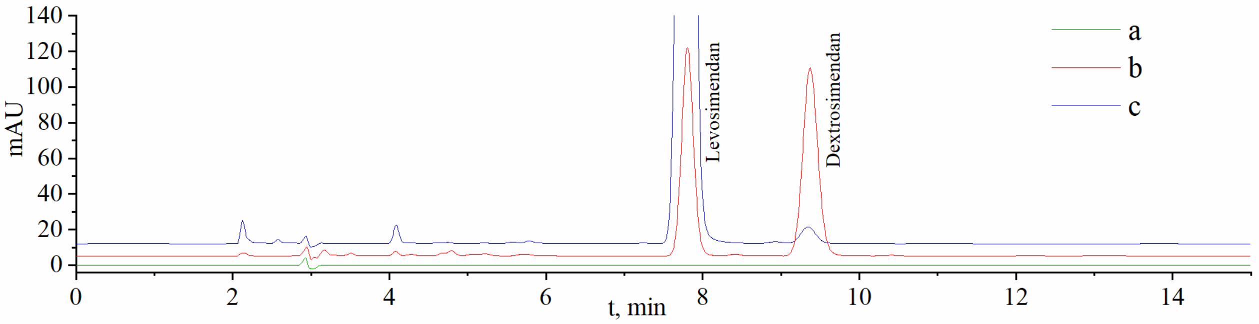


Figure 3. The (*R*)-enantiomer content test of the Levosimendan final sample, typical chromatograms: **a** – blank solution, **b** – system suitability solution, **c** – test solution

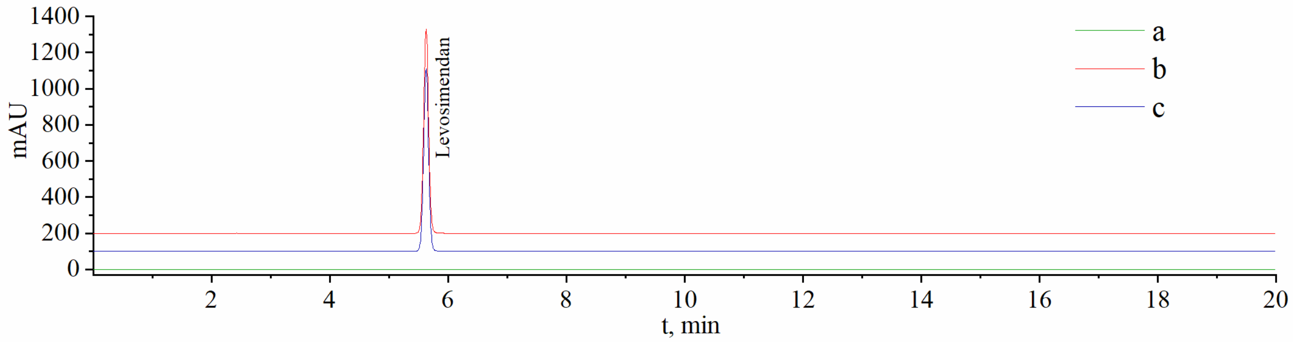


Figure 4. The assay test of the Levosimendan final sample typical chromatograms: **a** – reference solution, **b** – test solution, **c** – blank solution

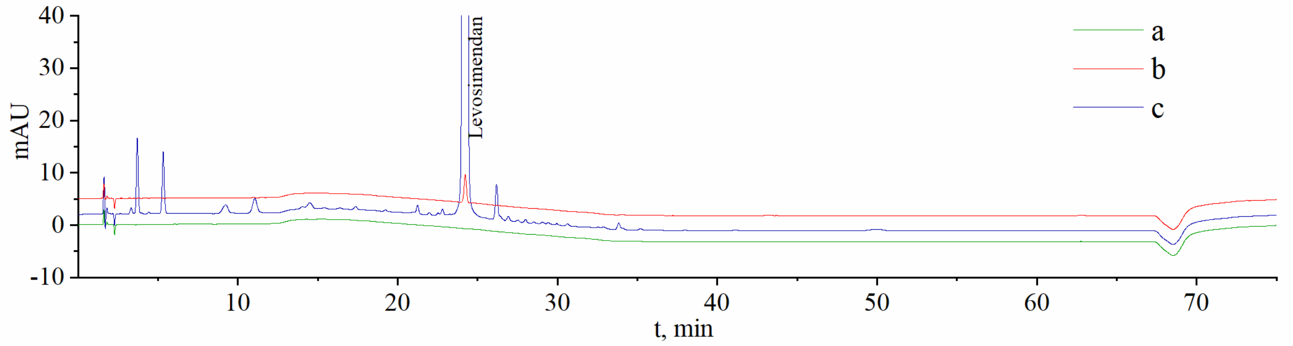


Figure 5. The assay test of the Levosimendan final sample typical chromatograms: **a** – reference solution, **b** – test solution, **c** – blank solution

6 injections of the reference solution chromatograms was not more than 2.0%, and the peak symmetry was not more than 2.0.

The test of related substances in the final sample was performed by the HPLC method on an Agilent Infinity II instrument and a Hypersil BDS C18 column (150 mm × 4.6 mm and 5 µm particle size) with the UV detection at 285 nm. The column temperature was set at 30°C. *Mobile phase A* was 0.2% triethylamine solution with pH 4.0 adjusted with trifluoroacetic acid, and *mobile phase B* was methanol. The gradient elution was carried out with a gradient program (0–10 min: 70% *A* – 30% *B*; 10–30 min: 40% *A* – 60% *B*, 30–65 min: 40% *A* – 60% *B*, 65–66 min: 70% *A* – 30% *B*, 66–75 min: 70% *A* – 30% *B*) and the flow rate at 1.0 mL min^{-1}.

The Levosimendan solution (0.003 mg mL^{-1}) was used as a reference solution. It was previously dissolved in DMSO, and then diluted with the solvent (0.05% trifluoroacetic acid solution in 50% methanol water solution). The solution of the final sample (2 mg mL^{-1}) was prepared for the test in the same way as the reference solution. All solutions were prepared in amber volumetric flasks, and the volumes of the injected solutions were 10 µL.

Typical chromatograms of blank, reference, and test solutions are shown in **Figure 5**. The chromatographic system was considered suitable if the relative standard deviation calculated for 6 injections of the reference solution chromatograms was not more than 5%, and the retention time was approximately 23 min.

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