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Synthesis of Isotope-labeled Aminoacids Contained in Servamycin Antibiotic

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Abstract—Approach was developed to a preparative synthesis of isotope-labeled aminoacids contained in servamycin IIB antibiotic. Glutamines labeled with ^{15}N, ^{13}C, and ^{2}H were prepared in 70–80% yield starting with the corresponding labeled glutamic acids under catalysis with the glutamine synthetase enzyme. ^{15}N-2-aminoisobutanoic acid and ^{15}N-isovaline were obtained by Strecker method in 65 and 31% yields respectively. All compounds synthesized were identified and characterized by NMR spectroscopy.

The study of peptide antibiotics labeled with stable isotopes with the use of isotope-sensitive methods is among the most informative procedures for investigation of interaction of these compounds with phospholipide membranes [1]. Therefore the preparation of isotope-labeled aminoacids and introducing them into the molecules of peptide antibiotics is indispensable step of such studies. Here we report on the developed approach to the preparative synthesis of aminoacids [L-glutamine, 2-aminoisobutanoic acid (Aib), and D-isovaline (D-Ival)] labeled with isotopes ^{2}H, ^{15}N, and ^{13}C in different positions aiming to introduce them into servamycin IIB molecule [2]. The servamycin IIB is a 16-membered peptide antibiotic efficient against both gram-positive and gram-negative bacteria that is capable to form ionic channels in the membranes of bacteria [3]. However the mechanism of formation and operation of these channels is not completely understood as yet.

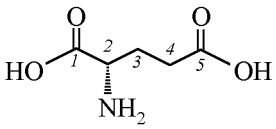
Introduction of isotopes into the rests of Aib and Ival of servamycin provides a possibility to study the antibiotic directly in the membrane phase and also to determine the part played by the above aminoacids in formation and operation of the ionic channels. The selection of Gln for isotope labelling was done under assumption that this rest plays a decisive role in opening and closing of the ionic channels formed by servamycin molecules [4].

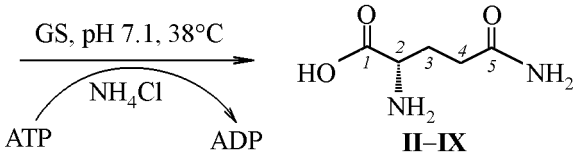
In the synthesis of labeled glutamines **II**–**IX** we applied a chemical enzymatic approach. The base of the method consisted in enzymatic conversion of labeled glutamic acids into the corresponding glutamines with the use of glutamine synthetase (GS) (Scheme 1).

(4,4-$^{2}H_2$)-Glu (**I**) was obtained from nonlabeled Glu via reaction of the chemical isotope exchange in 20% solution of ^{2}HCl in $^{2}H_2O$ (Scheme 2). The reaction was carried out at 100°C for 4 days. The completely labeled glutamic acid (**I**) was obtained in 75% yield.

The glutamine synthetase is known to be the catalyst of ATP-dependent conversion of glutamic acid into glutamine. However the ADP arising in the process operates as competitive inhibitor for the enzyme. To avoid this complication an alternative process may be used: ADP-dependent transformation

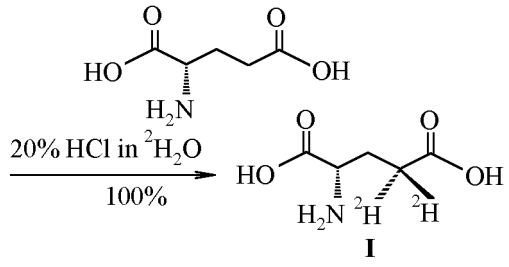
Scheme 1.





II–IX

Scheme 2.



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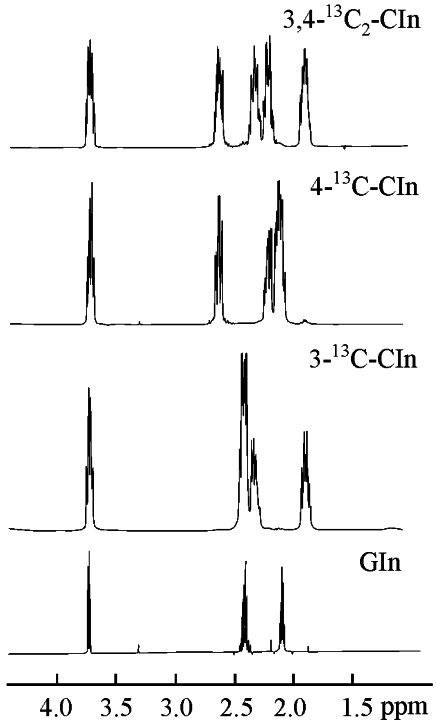


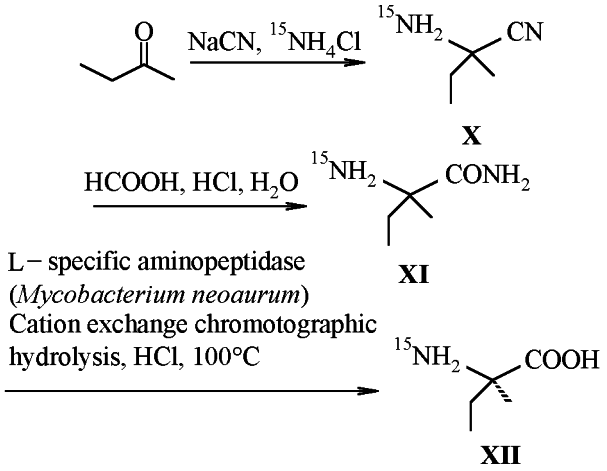
Fig. 1. ^{1}H NMR spectra of (^{13}C)-glutamines.

of phosphoenol pyruvate into pyruvate catalyzed by pyruvate kinase. However we believe this path to be economically unfeasible. Therefore to suppress the inhibition by ADP we used ATP in 3-fold excess with respect to the amino acid. The catalytic activity of the glutamine synthetase appears in the presence of bivalent metal cations in the reaction medium. As such we used magnesium cations (Mg^{2+}) [5]. In the synthesis of (5-^{15}N)-Gln (**VI**) as the nitrogen source was used $^{15}NH_4Cl$. The reactions were carried out in 80 mM imidazole buffer (pH 7.1) at 38°C for 24 h. The isolation of pure labeled glutamines was carried out by ion-exchange chromatography on a cation-exchanger AG 5OW-X8 (form NH_4^+) to remove all positive ions, and then on an anion-exchanger AG1-X8 (form Ac^-) to remove all negative ions. Thus in the course of this study we succeeded in developing conditions for preparation of fully labeled glutamines in 70–80% yield (Table 1).

The ^{1}H NMR spectra of (^{2}H)-Gln are presented in Table 1. The analysis of the spectrum of compound **I** as compared with that of unlabeled glutamic acid demonstrates the lack of a multiplet in the 2.5 ppm region and appearance of a doublet from H^3 proton; these facts evidence the replacement of H_4 proton by deuterium. Similar features are observed in the spectrum of compound **II**. The disappearance of the multiplet at 2.12 ppm in the spectrum of compound **III** and also singlets from the protons H_2 and H_4 indicate the replacement by deuterium of the proton H_3. In the spectrum of compound **IV** is lacking the signal at 3.74 ppm corresponding to H_2 proton.

The ^{1}H NMR spectra of the prepared (^{13}C)-glutamines are presented on Fig. 1. As seen, the signal of H_2 proton at 3.76 ppm appears as a multiplet instead of a triplet due to the coupling with the ^{13}C isotope. Moreover, the presence of the ^{13}C in position 3 (or) 4 provides the splitting of the geminal protons H_3 and H_4 (J 128 Hz). The rest coupling constants in the (3,4-$^{13}C^2$)-Gln molecule are listed in Table 2.

Quite a number of procedures in known nowadays for the preparation of α,α-dialkylated amino acids. Most of the methods are based on intermediate synthesis of α-aminonitriles by Strecker reaction [6]. In this study we used this approach for preparative synthesis of (^{15}n)-D-Ival (**XII**) and Aib (**XIII**) proceeding from mixtures of the respective ketones, ^{15}N-ammonium chloride, and sodium cyanide.



The synthesis of D-Ival (**XII**) consists of three stages. In the first stage (α-^{15}N)-D,L-aminoisovaleronitrile (**X**) was obtained in the quantitative yield from 2-butanone and water solution of NaCN and $^{15}N_4Cl$ taken at the molar ratio 2 : 2 : 1. The prepared aminonitrile **X** was subjected to mild hydrolysis with formic acid saturated with HCl in the presence of the equimolar amount of water. As a result arose (α-^{15}N)-D,L-aminoisovalerylamide (**XI**). In the second stage amide (**XI**) was subjected to stereoselective hydrolysis effected by bacteria Mycobacterium neoarum ATTC 25795 [7] that contained L-specific aminopeptidase. As a result formed the L-isomer of the labeled Ival whereas the (α-^{15}N)-D-aminoisovaleryl-

amide remained intact. Then the cells were removed by centrifugation, and the mixture of (^{15}N)-L-Ival and (α-^{15}N)-D-aminoisovalerylamide was separated on cation-exchanger Amberlite CG 50. At the last stage the optically pure D-amide was hydrolyzed with conc. HCl to obtain compound **XII** in overall yield 31%. The stereoselectivity of the procedure according to the data of HPLC was over 99.5%.

Under the same conditions compound **XIII** was obtained from acetone in low yield (20%) and with partial loss of the label (isotope enrichment did not exceed 75%) (Fig. 2b). It seemed unreasonable since the chemical difference between acetone and 2-butanone is slight. However acetone is miscible with water whereas the 2-butanone forms a separate phase in the reaction mixture. Therefore we presumed that the presence of the second phase is important for this reaction. Actually, when the reaction was carried out in the presence of a solvent unmiscible with water the resulting product **XIII** was fully labeled (Fig. 2). We also observed that the yield of the product depended on the polarity of solvent forming the second phase. For instance, at the use of hexane the yield of compound **XIII** was 32%, with ethyl ether 36%, with ethyl acetate 40%, and with dichloromethane 65%.

The synthesized compounds labeled with ^{15}N were characterized with ^{1}H NMR spectra (Figs. 2, 3). The presence of ^{15}N was proved by ^{15}N NMR spectra, and the degree of isotope enrichment was confirmed by mass spectrometry. As seen from Fig. 2 and 3, in the region of 1.57 ppm instead of a singlet appeared a doublet due to the coupling between the protons of the α-methyl groups with ^{15}N [J(CH_3, ^{15}N) was 2.8 Hz in Ival (**XII**) and 2.6 Hz in Aib (**XIII**)]. In the spectrum of compound **XII** two multiplets should be indicated at 2.00 and 1.90 ppm belonging to the methylene protons. This splitting of signal corresponds to nonequivalence of the protons in the methylene group. This fact is an indirect evidence of the conformational rigidity of Ival molecule.

Thus as a result of this study applying the modern enzymatic and chemical-enzymatic approach were obtained at a preparative scale and characterized various aminoacids labeled with stable isotopes (Gln, Aib, Ival) that were further successfully introduced into the molecule of servamycin IIB.

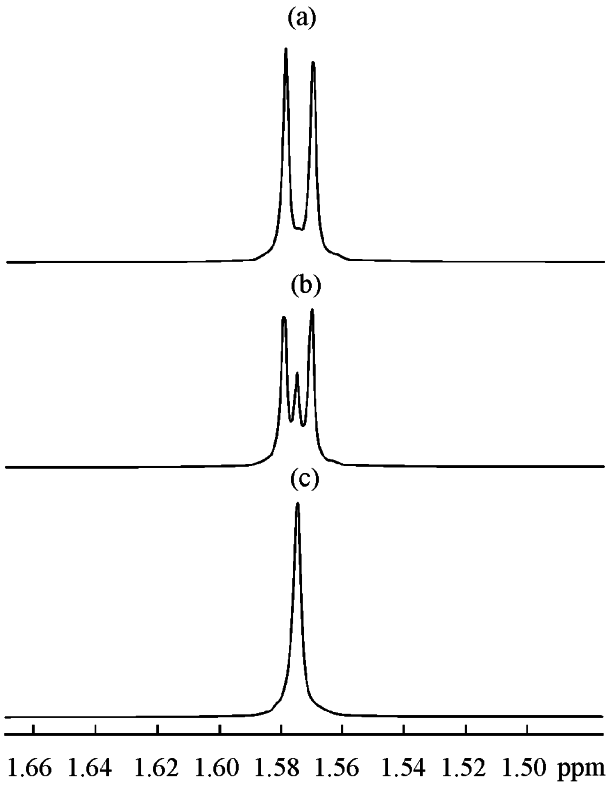


Fig. 2. ^{1}H NMR spectra of(^{15}N)-Aib, prepared (a) in two-phase and (b) in single-phase systems, and (c) of unlabeled Aib.

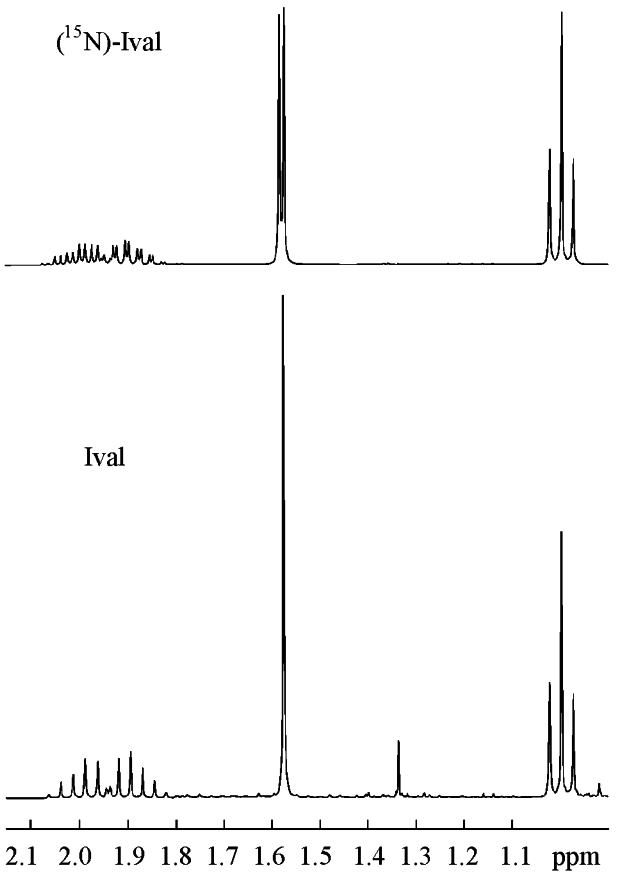


Fig. 3. ^{1}H NMR spectra of unlabeled and labeled (^{15}N)-isovaline.

Table 1. Yields and data of ^{1}H NMR spectra of glutamines labeled with ^{2}H, ^{15}N, and ^{13}C

Compound no.	Name of compound	Yield, %	mp, °C (water)	Chemical shift, δ, ppm		
				H^2	H^3	H^4
–	L-Glutamic acid	–	226 [8]	3.94 t	2.06 m	2.50 m
I	(4,4-$^2H_2$)-L-Glutamic acid	75	225–227	3.93 t	2.02 m	–
–	L-Glutamine	–	185 [8]	3.74 t	2.12 m	2.42 m
II	(4,4-$^2H_2$)-L-Glutamine	75	184–186	3.76 t	2.10 d	–
III	(3,3-$^2H_2$)-L-Glutamine	74	183–185	3.74 s	–	2.43 s
IV	(2-^{2}H)-L-Glutamine	79	186–188	–	2.12 m	2.44 m
V	(α-^{15}N)-L-Glutamine	71	184–186	3.79 t	2.17 m	2.43 m
VI	(5-^{15}N)-L-Glutamine	80	182–184	3.77 t	2.15 m	2.45 m
VII	(3-^{13}C)-L-Glutamine	76	185–187	3.73 m	2.10[a] m	2.43 m
VIII	(4-^{13}C)-L-Glutamine	74	183–185	3.75 m	2.11 m	2.40[a] m
IX	(3,4-$^{13}C^2$)-L-Glutamine	71	184–186	3.76	2.12[a] m	2.43[a] m

[a] For coupling constants see Table 2.

Table 2. Spin-spin coupling constants ($J_{A,X}$, Hz) in molecules of (3-^{13}C-) (**VII**), (4-^{13}C-)- (**VIII**) and (3,4-$^{13}C^2$)-Gln (**IX**)

X	H^2	H^3	H^4	$^{13}C^3$	$^{13}C^4$
H^2	–	4.7[a]	–	1.3	–
H^3	4.7	–	4.5[b]	128	2.0
H^4	–	4.5	–	2.0	128
$^{13}C^3$	1.3	128	2.0	–	–
$^1XC^4$	–	2.0	128	–	–

EXPERIMENTAL

In the study were used 3,3-$^2H_2$-, 2-^{2}H-, 3-^{13}C-, 4-^{13}C-, 3,4-$^{13}C_2$- and α-^{15}N-glutamic acids kindly provided by Leiden University (the Netherlands), $^{15}NH_4Cl$ (Cambridge Isotope Laboratories), ^2H_2O (Aldrich), and also Russian reagents and solvents of high purity. Enzymatic processes were carried out at pH 7.1 and 38°C with the use of enzyme glutamine synthetase (GS, E.C.6.3.1.2.) Escherichia coli from Sigma Co (USA). The monitoring of reactions was performed by thin-layer chromatography of the plates DC-Alufolien, Kieselgel Go, F 254 (Merck) in the following solvent systems: 2-propanol–H_2O–HCOOH, 20 : 5 : 1 (A); 2-propanol–ammonia, 7 : 3 (B). Development was performed with ninhydrin (0.5% solution in acetone). HPLC was carried out on Knauer instrument (Germany) using a column 4ξ250 mm, Spherisorb ODS-2 (C18), flow rate 0.4 ml/min, gradient of methanol concentration from 15% in the mixture methanol–sodium acetate (15 : 85) to 100%. Ion-exchange chromatography was carried out on columns 50 × 80 mm and 100 × 25 mm packed with ion exchangers AG 50 W-X8 (NH_4^+ form) and AG-1X8 (Ac form) respectively (Bio-Rad Laboratories). The ^{1}H NMR spectra were registered on Bruker WM-300 spectrometer operating in Fourrier-transform pulse mode at 300 MHz, internal reference TMS (δ 0.00 ppm) in D_2O or CD_3OD, and Ival spectrum was recorded in CD_3OD on a spectrometer with the operating frequency 600 MHz. The ^{15}N NMR spectra were registered on Bruker DMX-600 instrument at 30.4 MHz, internal standard ammonium nitrate-^{15}N (δ 19.0 ppm).

L-glutamic-4,4-$^2H_2$ acid (I). Into an ampule was charged 1 g (6.8 mmol) of L-glutamic acid and 10 ml of 20% DCl solution in D_2O. The ampule was cooled in liquid nitrogen and sealed in a vacuum. The reaction was carried out for 4 days at 100°C. Then the reaction mixture was cooled, and 25 ml of ethanol was added thereto. The separated precipitate was filtered off and washed with ethanol. Yield of aminoacid I 0.75 g (75%), mp 225–227°C (226°C [8]), colorless crystals R_f 0.42 (A). The data on ^{1}H NMR spectrum are given in Table 1.

Synthesis of labeled glutamines II–IX (general procedure). In 50 ml of 80 mM imidazole buffer (pH 7.1) were dissolved 0.5 mmol of labeled glutamic acid, 1.5 mmol of ATP, 1 mmol of $^{15}NH_4Cl$, and 4 mmol of $MgCl_2$–6 H_2O; then was added thereto 2.5 mg (70 units) of glutamine synthetase, and the mixture was maintained for 24 h at 38°C. The solu-

tion obtained was passed in succession through a column 50 × 80 mm packed with cation-exchanger AG 50 W-X8 W-X8 (NH_4^+ form) and through a column 100 × 25 mm packed with anion-exchanger AG-1X8 (Ac^- form), eluent water. The eluent was evaporated, and the residue was subjected to liophilic drying. R_f is 0.22 (A) for all the glutamates obtained. Yields, melting points, and ^1H NMR spectral data of compounds **II–IX** are given in Table 1.

Isovaline-(^{15}N) (XII). In 15 ml of water was dissolved 0.2 g (43 mmol) of sodium cyanide and 1.16 g (21.5 mmol) of $^{15}NH^4Cl$, and 2 ml (43 mmol) of 2-butanone was added thereto. The reaction mixture was stirred for 12 h at room temperature in a sealed ampule. The formed aminonitrile **X** was extracted into dichloromethane, the extract was dried with $MgSO_4$, and the solvent was distilled off. The yield of compound X 2.12 g (quantitative). ^1H NMR spectrum (300 MHz, CD_3OD), δ, ppm: 1.70 m [2H, CH_2, ^3J(CH, $^{\beta}CH_3$) 7.5 Hz, ^3J(CH, ^{15}N) 1.4 Hz], 1.46 d [3H, $^{\alpha}CH_3$, $^3J(^{\alpha}CH_3, ^{15}N)$ 3.0 Hz], 1.10 t [3H, $^{\beta}CH_3$, $^3J(^{\beta}CH_3, CH_2)$ 7.5 Hz]. Aminonitrile **X** obtained was subjected to mild hydrolysis in 20 ml of formic acid saturated with hydrogen chloride and containing also 0.38 ml (21.5 mmol) of water. The reaction proceeded for 2 h. The reaction mixture was then evaporated, and amide **XI** thus obtained was crystallized from 2-propanol. Yield of compound **XI** 2.24 g (90%). ^1H NMR spectrum (300 MHz, CD_3OD), δ, ppm: 2.00 m [1H, CH^A, ^3J(CH, $^{\beta}CH_3$) 7.5 Hz, ^3J(CH, ^{15}N) 3.6 Hz], 1.88 m [1H, CH^B, ^3J(CH, $^{\beta}CH_3$) 7.5 Hz, ^3J(CH, ^{15}N) 2.1 Hz], 1.58 d [3H, $^{\alpha}CH_3$, $^3J(^{\alpha}CH_3, ^{15}N)$ 2.8 Hz], 1.00 t [3H, $^{\beta}CH_3$, $^3J(^{\beta}CH_3, CH_2)$ 7.5 Hz]. 2.23 g (19.3 mmol) of amide **XI** was dissolved in 19.3 ml of water (pH was adjusted at 8.5 with addition of 5 M solution of KOH). Then was added 150 mg of cells Mycobacterium neoarum ATTC 25795, and the mixture was maintained at 38°C for 20 h. Afterwards the cells were removed by centrifugation, and the solvent was evaporated. The separation of L-isovaline and D-aminoisovalerylamide-(α-^{15}N) was performed on a column 5 × 20 cm packed with cation-exchanger Amberlite CG 50. Elution with water afforded L-isovaline, and the pure labeled D-aminoisovalerylamide was eluted with 1 M ammonia solution. Overall yield 31%. The separated amide was hydrolyzed with conc. HCl within 2 h. The solvent was distilled off, the dry residue was dissolved in 2-propanol, and the solution was evaporated. The latter procedure was repeated thrice. Yield of labeled isovaline (**XII**) 0.68 g (100%), R_f 0.56 (B). ^1H NMR spectrum(600 MHz, CD_3OD), δ, ppm: 2.00 m [1H, CH^A, ^3J(CH, $^{\beta}CH_3$) 7.5 Hz, ^3J(CH, ^{15}N) 3.6 Hz], 1.90 m [1H, CH^B, ^3J(CH, $^{\beta}CH_3$ 7.5 Hz, ^3J(CH, ^{15}N) 2.1 Hz], 1.57 d [3H, $^{\alpha}CH_3$, $^3J(^{\alpha}CH_3, ^{15}N)$ 2.8 Hz], 1.00 t [3H, $^{\beta}CH_3$, $^3J(^{\beta}CH_3, CH_2)$ 7.5 Hz]. ^{15}N NMR spectrum (30.4 MHz, CD_3OD), δ_N, ppm: 48.63.

Aminoisobutanoic-(^{15}N) acid (XIII). To a solution of 2 g (43 mmol) of sodium cyanide and 1.16 g (21.5 mmol) of $^{15}NH_4Cl$ in 7.5 ml of water and 7.5 ml of dichloromethane was added 2 ml (43 mmol) of acetone. The reaction mixture was stirred in a sealed ampule for 12 h at room temperature. then the reaction mixture was hydrolyzed for 2 h with conc. HCl at 100°C. The solvent was distilled off, the dry residue was dissolved in 2-propanol, and the solution was evaporated. The latter procedure was repeated several times. Yield of compound **XIII** (**XIII**) 1.41 g (65%), mp 335–337°C (335°C [8]), colorless crystals, R_f 0.50 (B). ^1H NMR spectrum (300 MHz, CD_3OD), δ, ppm: 1.57 d [6H, $2CH_3$, $^3J(CH_3, ^{15}N)$ 2.6 Hz]. ^{15}N NMR spectrum (30.4 MHz, CD_3OD), δ_N, ppm: 48.63.

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