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ON DIFFERENCES BETWEEN RACEMIC AND ENANTIOMERICALLY PURE FORMS OF AZIRIDINE-2-CARBOXAMIDE*

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Synthesis, X-ray, and cytotoxicity studies of (S)- and (R)-aziridine-2-carboxamide (Leakadine) are described. X-ray data for the enantiomerically pure form are compared with those for racemic aziridine-2-carboxamide in order to explain the 21°C large melting point difference between both series. It was found that despite their overall low cytotoxicity (S)-aziridine-2-carboxamide is slightly more cytotoxic than (R)-aziridine-2-carboxamide.

Keywords: aziridine-2-carboxamide, chiral aziridines, Leakadine.

Aziridines are rather reactive constrained heterocycles. More than a hundred compounds from the aziridine class have shown certain biological activity [1]. In many cases, this activity is based on the strong alkylating properties of aziridines. Therefore, many representatives of the aziridine series possess distinct cytotoxicity rather than selective biological activity. On the other hand, there are several classes of aziridines that have selective activity. These are the natural alkaloids mitomycins [2], peptides madurastatin and miraziridine [3], anticancer drug azinomycin A [4] and others. Additionally, derivatives of aziridine carboxylic acid may act as neoplasm inhibitors and therefore are considered as useful anticancer drugs [5, 6]. The latter class includes Azimexon, Imexon, and aziridine-2-carboxamide (Leakadine, **1**) [7-9]. In the case of Imexon, each of its enantiomers has been studied separately [10]. On the other hand, racemic amide **1** is known since 1957 as a chemical entity [11]. The enantiopure forms of compound **1** have been prepared, but their crystal structures and other properties have not been studied in detail. Thus, Nakajima *et al.* have obtained isomer (*S*)-**1** *via* (2*S*)-aziridine carboxylic acid benzyl ester [12], but Jähnisch *et al*. produced isomer (*R*)-**1** in a kinetic resolution experiment using a sophisticated derivative of (*R*)-carvone [13].

*Dedicated to Professor Ivars Kalvinsh on the occasion of his 65th birthday.

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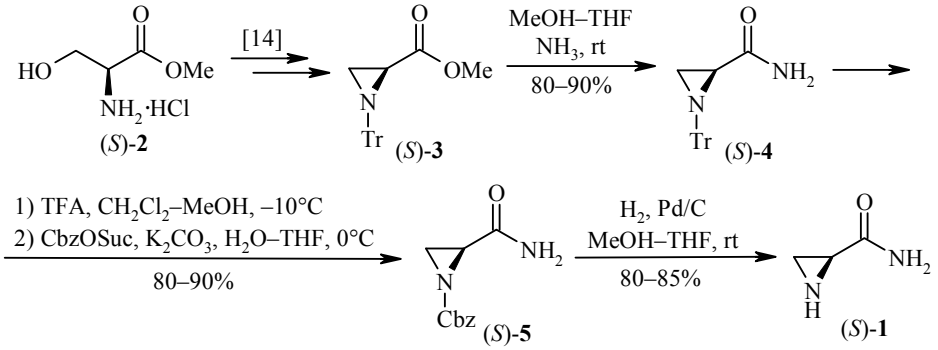
We report here a practical synthesis of (*S*)-Leakadine ((*S*)-**1**) and (*R*)-Leakadine ((*R*)-**1**), comparative X-ray studies of enantiomerically pure isomer (*R*)-**1** and racemic compound (±)-**1**, and the initial cytotoxicity results of the aforementioned products. In order to prepare sufficiently large quantities of enantiomerically pure forms of Leakadine (**1**), we have based our synthetic plan on the well-established synthesis of (*R*)- and (*S*)-*N*-tritylaziridine-2-carboxylic acid methyl esters ((*S*)-**3** and (*R*)-**3**). These latter were obtained from (*R*)- and (*S*)-serine methyl ester hydrochloride (**2**), respectively [14]. The syntheses occur identically in (*R*)- and (*S*)-series, and only the latter is depicted in the scheme. Ammonolysis of ester (*S*)-**3** with ammonia followed by exchange of *N*-protecting group produced *N*-benzyloxycarbonyl-protected intermediate (*S*)-**5** [15]. It should be noted that both intermediates (*S*)-**4** [16] and (*S*)-**5** are relatively stable, but deprotection of the latter *via* hydrogenation produces crude (*S*)-**1** in a much more convenient way [17]. Moreover, detritylation in the amide stage is preferable over a similar step in the ester (*S*)-**3** stage as the proton H-2 in the latter compound is more labile and thus more prone to racemization. The overall process is easily scalable and permits the reproducible synthesis of both enantiomeric forms of Leakadine (**1**) on a gram scale. HPLC analysis on the chiral stationary phase revealed that both isomers (*S*)-**1** and (*R*)-**1** are of excellent enantiomeric purity (*ee* > 99.8%). HPLC conditions were developed with racemic Leakadine ((±)-**1**) as a standard substance, which was prepared by combining previously published procedures of synthesis of aziridine carboxylic acid methyl ester [9] and its aminolysis [18].



Melting points of enantiomerically pure (*S*)-**1** or (*R*)-**1** crystallized from methanol (153°C) and racemic Leakadine ((±)-**1**) (132°C, MeOH) were determined by differential scanning calorimetry. As expected, isomers (*S*)-**1** and (*R*)-**1** possessed identical melting point values, which differed significantly from that of racemic amide (±)-**1.** This prompted us to perform more detailed studies by single crystal X-ray diffraction analysis. Here we report the first X-ray studies of both racemic Leakadine (**1**) and (*R*)-Leakadine ((*R*)-**1**). Monocrystals of excellent quality were obtained also for isomer (*S*)-**1**, but, as expected, they revealed a diffraction pattern identical to that of isomer (*R*)-**1** and, therefore, were not studied further.

The molecular structure of the isomer (*R*)-**1** with atomic numbering scheme is presented in Figure 1. The crystallographic parameters of both structures (±)-**1** and (*R*)-**1** are equal within error limits. However, the molecular packing in crystals of the racemate (±)-**1** differs significantly from the packing of the pure enantiomer (*R*)-**1**. Figure 2 shows the packing diagram in the crystal structure of amide (±)-**1**. The packing diagram of compound (*R*)-**1** is illustrated in Figure 3. Molecules of isomer (*R*)-**1** form the axial orthorhombic crystal lattice (space group $P2_12_12_1$), whereas racemate (±)-**1** gives the centrosymmetric monoclinic lattice (space group $P2_1/c$). In both crystal structures all hydrogens of the NH groups participate in the intermolecular bifurcated hydrogen bonds.

The hydrogen bonds form a three-dimensional net in the crystal structures. In the crystal structure of racemate (±)-**1** there are moderate N(6)−H···O and N(6)−H···N hydrogen bonds and weak N(1)−H(1)···O(5) bonds. In the crystal structures of isomers (*R*)-**1** and (*S*)-**1** there are moderate N(6)−H···O and N(6)−H···N bonds and very weak N(1)−H(1)···O(5) hydrogen bonds. On the other hand, the intermolecular distance

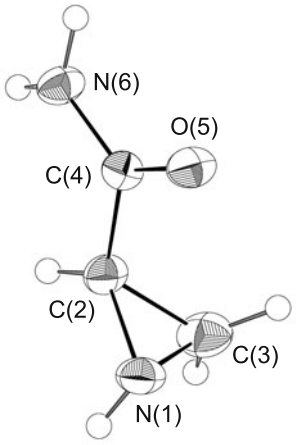


Fig. 1. Molecular structure of the isomer (*R*)-**1** with representations of the atomic thermal vibration ellipsoids at 50% probability.

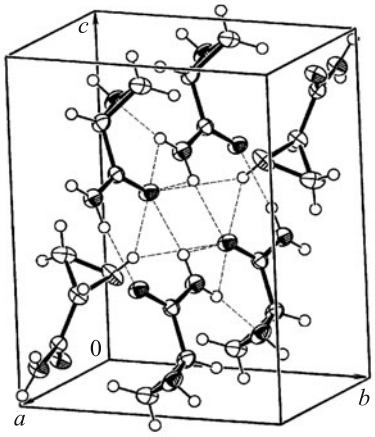


Fig. 2. The packing arrangement in the unit cell of racemate (±)-**1** (mp 132°C) showing the intermolecular hydrogen bonds.

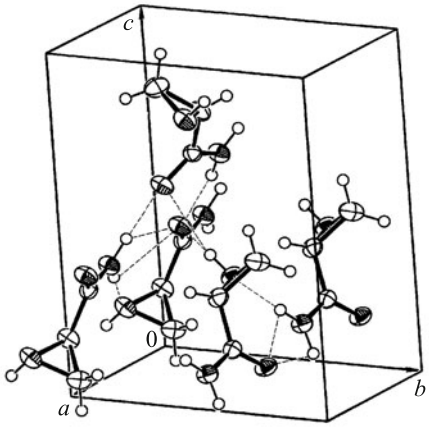


Fig. 3. The packing arrangement in the unit cell of isomer (*R*)-**1** (mp 153°C) showing the intermolecular hydrogen bonds.

C(3)···C(3) equal to 3.366(3) Å in racemate (±)-**1** destabilizes the crystal as the sum of Van der Waals' radii for carbon atoms is 3.4–3.5 Å. The crystal packing of enantiopure forms show the distance C(3)···C(3) equal to 3.652(4) Å. This may explain the different melting points for the racemate and the pure enantiomers.

Additionally, one can assume that the melting points of racemic and enantiomerically pure Leakadine (**1**) are determined by entropy factors. The thermodynamic expression $T = Q/S$, where Q is heat of the phase transition and S is its entropy, can be written for the phase transition temperature, T. This means that the relatively low entropy of crystals of isomers (*R*)- and (*S*)-**1** leads to an increase in melting point. The entropy value for the monoclinic structure of racemate (±)-**1** is higher than for the orthorhombic structure of isomer (*R*)-**1**, and this situation results in the decreased melting point of racemate (±)-**1** compared to isomers (*R*)- and (*S*)-**1**.

The cytotoxic activity of isomers (*R*)-**1**, (*S*)-**1** and racemate (±)-**1** has been studied. The following cell lines were used in the current study: large-cell lung carcinoma cell line NCI-H460 (ATCC number HTB-177, Fig. 4), human pancreatic adenocarcinoma BxPC-3 (ATCC number CRL-1687, Fig. 5), and normal human umbilical vein endothelial cells (HUVEC, ATCC number PCS-100-010, Fig. 6). All cells were propagated under conditions suggested by the provider. Each compound was tested on three cell lines at least in three

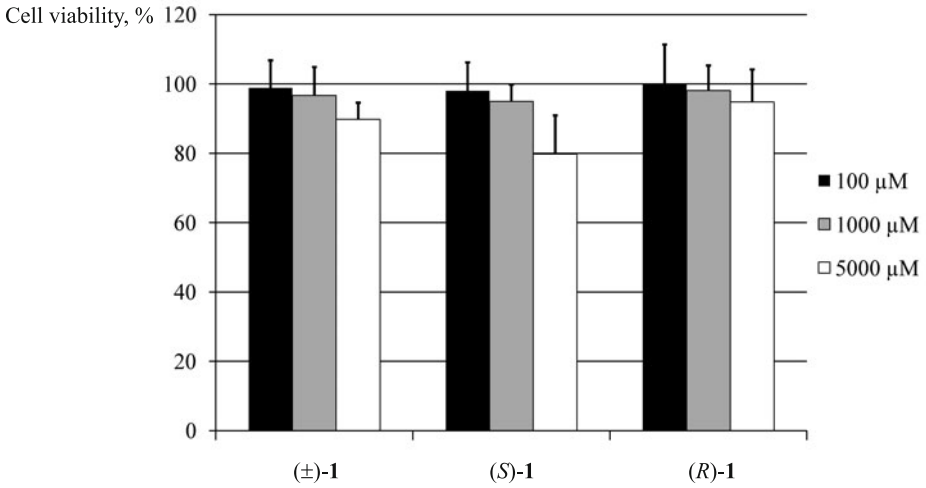


Fig. 4. Inhibition of NCI-H460 lung cancer cell proliferation by Leakadine isoforms in the range of effective doses.

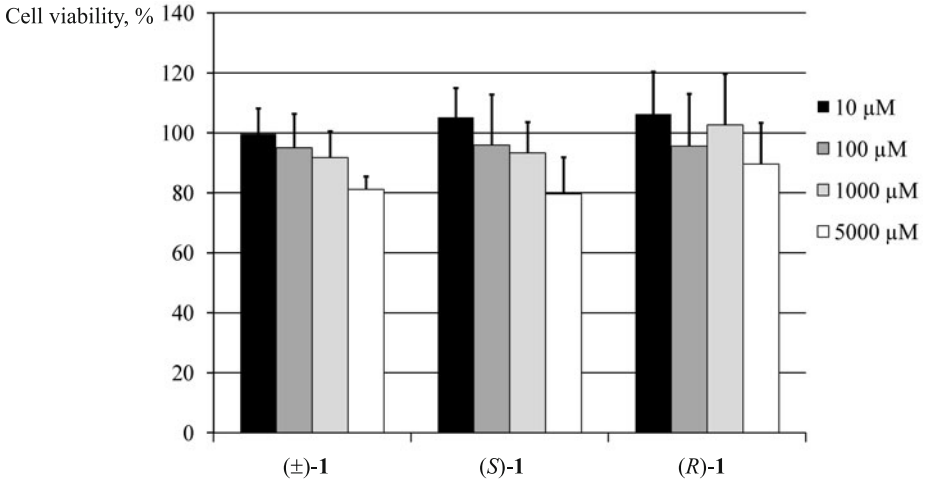


Fig. 5. Inhibition of BxPC-3 pancreatic adenocarcinoma cell proliferation by Leakadine isoforms in the range of effective doses.

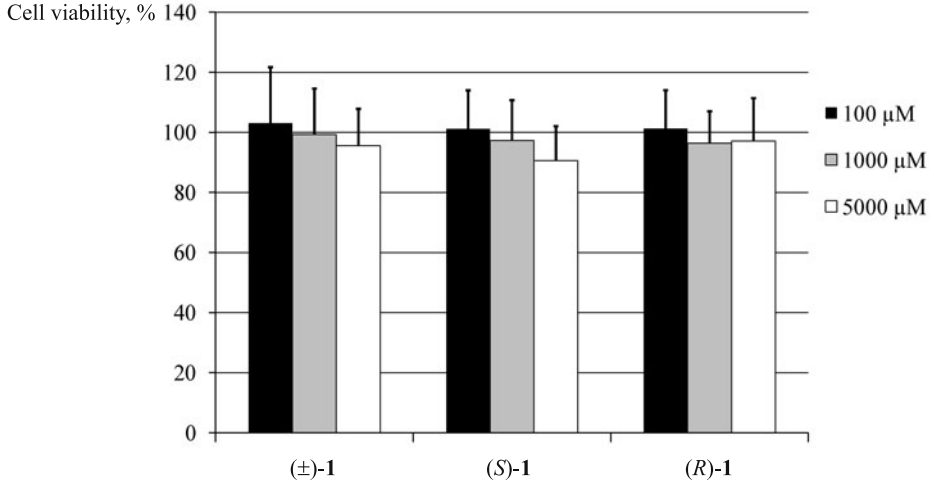


Fig. 6. Inhibition of normal human cells (human umbilical vein cell line HUVEC)proliferation by Leakadine isoforms in the range of effective doses.

concentrations. Effective concentrations were obtained experimentally in preliminary test series where the effect on ascending concentrations of drugs on cell proliferation (toxicity) was measured using MTT methodology [19]. Every single test was performed in at least five parallels.

MTT tests determine the toxicity of compounds based on cell proliferation. It measures the mitochondrial activity of cells in a tested population. It is a widely used effective and inexpensive test in drug development that measures the effect of tested compounds on mitochondrial metabolism [19]. According to available data Leakadine (**1**) is an immunostimulator that elevates the levels of antitumor factors of the immune system – T helper cells and locally synthesized antibodies – and indirectly reduces the activity of T suppressors that inhibit immunological responses in growing neoplasms [20]. Therefore one can expect a relatively weak effect of isomers (*R*)-**1**, (*S*)-**1**, and racemate (±)-**1** on cancer cell proliferation (MTT assay). Nevertheless, this is the first report on comparative cytotoxicity studies of isomers (*R*)-**1**, (*S*)-**1**, and racemate (±)-**1** on the given cell lines, and experimental results show that different Leakadine (**1**) forms behave slightly differently on all tested cell lines. It is interesting to note that isomer (*R*)-**1** shows less toxicity. This means that this Leakadine (**1**) enantiomer will produce fewer side effects if applied in the systemic treatment of cancer. Further studies on the impact of enantiomerically pure forms of Leakadine (**1**) on the immune system will be reported elsewhere.

In summary, we have developed a straightforward, reproducible, and scalable method for the synthesis of both enantiomers of aziridine-2-carboxamide (Leakadine). We have found that there are significant differences between the crystal packing of its enantiomerically pure and racemic forms. At the macroscopic level this is manifested by a melting point difference as high as 21°C. An initial MMT assay revealed that the (*S*)-isomer is slightly more cytotoxic than the (*R*)-isomer.

EXPERIMENTAL

IR spectra were recorded on a Perkin-Elmer Spectrum BX FT-IR instrument in KBr discs. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer (300 and 75 MHz, respectively); solvent residual signal or MeOH were used as internal standard [21]. HPLC was performed on an Agilent Technologies 1200 Series chromatograph with a UV-Vis diode array detector (220 nm); reverse phase C18, 3.5 μm, 4.6×150 mm, 0.8 ml/min; eluent A: 0.01 M KH_2PO_4 in water containing 6% MeCN; eluent B: MeCN; gradient: 20 to 90% B in 10 min, 90% B 5 min. Enantiomeric excess of enantiomerically pure Leakadine (**1**) was determined by HPLC (Chiralpak IC 46 × 250 mm), eluent system: 2-PrOH–hexane, 1:1, in isocratic mode, flow rate 0.35 ml/min; UV detector at 210 nm. Melting points of compounds (*S*)-**4**, (*R*)-**4**, (*S*)-**5**, and (*R*)-**5** were determined on a Stuart SMP10 melting point apparatus and are uncorrected. Melting points of compounds (*S*)-**1**, (*R*)-**1**, and (±)-**1** were determined by an indium-calibrated (mp 156.6°C) differential scanning calorimeter Mettler 300 (Mettler-Toledo AG). Specific rotation data were recorded on an Atago AP-300 polarimeter. Progress of the reactions was monitored by TLC using Merck Silica Gel 60 F_{254} plates.

Reactions were carried out using solvents and reagents dried according to standard procedures. Isolated yields refer to chromatographically homogeneous substances.

The cytotoxicity measurements were performed following Mossman *et al*. [19]. MTT indicates coloration by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide. The optical density in the biological tests in 96-well microtiter plates was determined using a Tetretek Multiscan MC C/340 horizontal spectrophotometer.

(-)-(2*S*)-*N*-(Triphenylmethyl)aziridine-2-carboxamide ((*S*)-4). Gaseous NH_3 was bubbled through a solution of ester (*S*)-**3** [14] (35.0 g, 0.102 mol) in MeOH (1000 ml) and THF (50 ml) at 0°C for 1 h. The resulting reaction mixture was stirred for 36 h at ambient temperature followed by evaporation of the solvents. Et_2O (100 ml) was added to the solid residue, and the resulting suspension was stirred for 2 h at ambient temperature and filtered. Yield 30.5 g (91%). In the series consisting of eight experiments isolated yields of amide (*S*)-**4** varied from 80 to 90%. Mp 150-153°C. $[\alpha]_D^{22}$ –63° (*c* = 2.00, $CHCl_3$). IR spectrum, ν, cm^{-1}: 3453,

3288, 3155, 3055, 2924, 2853, 1659, 1600, 1445. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.44-7.39 (6H, m, H Ph); 7.32-7.22 (9H, m, H Ph); 6.67 (1H, br. s) and 5.38 (1H, br. s, $CONH_2$); 2.07 (1H, d, ^3J = 2.7, 3-CH_A); 1.98 (1H, dd, ^3J = 6.6, ^3J = 2.7, 2-CH); 1.51 (1H, d, ^3J = 6.6, 3-CH_B). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 173.8; 143.2; 129.2; 127.8; 127.2; 74.5; 33.9; 29.9. Found, %: C 80.51; H 6.08; N 8.63. $C_{22}H_{20}N_2O$. Calculated, %: C 80.46; H 6.14; N 8.53.

(+)-(2*R*)-*N*-(Triphenylmethyl)aziridine-2-carboxamide ((*R*)-4) was prepared from ester (*R*)-**3** in a similar manner to that described above. In the series consisting of six experiments isolated yields of amide (*R*)-**4** varied from 80 to 90%. Mp 150-153°C. $[\alpha]_D^{22}$ 63° (*c* = 2.00, $CHCl_3$). Other analytical data of amide (*R*)-**4** are identical to those of isomer (*S*)-**4**.

(-)-(2*S*)-*N*-Benzyloxycarbonylaziridine-2-carboxamide ((*S*)-5). Trifluoroacetic acid (96.3 ml, 1.3 mol) was added to a solution of (*S*)-**4** (21.3 g, 64.9 mmol) in CH_2Cl_2 (85 ml) and MeOH (102 ml) at -10 to -15°C. The resulting reaction mixture was stirred at -10°C for 10 h (TLC control, eluent $CHCl_3$–MeOH, 19:1). Crushed ice (300 g) was added and the organic phase was evaporated under reduced pressure (bath temperature ≤ 30°C). The precipitate (Ph_3COH + Ph_3COMe) was filtered and washed with water (20 ml). The filtrate was cooled to 0°C and carefully neutralized at this temperature with solid K_2CO_3 (52 g) until pH 8-9. The resulting basic cold aqueous solution was diluted with THF (200 ml) and solution of *N*-(benzyloxycarbonyl-oxy)succinimide (17.0 g, 68.4 mmol) in THF (130 ml) was added at -5 to 0°C with vigorous stirring. The pH level of the resulting mixture was controlled at 8-9 during the full course of the reaction. After stirring for 4 h at 0°C the reaction mixture was extracted with $CHCl_3$ (3×200 ml). The combined organic layer was washed with brine (100 ml), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Et_2O (80 ml) was added to the solid residue, and the resulting suspension was stirred for 1 h at ambient temperature and filtered. Yield 12.9 g (91%). In the series consisting of eight experiments isolated yields of isomer (*S*)-**5** varied from 80 to 90%. Mp 125-126°C. $[\alpha]_D^{23}$ -95° (*c* = 2.0, $CHCl_3$). IR spectrum, ν, cm^{-1}: 3394, 3321, 3272, 3213, 3065, 3028, 3008, 2935, 1712, 1677, 1458, 1433, 1398, 1342, 1306, 1200. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm (*J*, Hz): 7.94 (1H, br. s) and 7.46 (1H, br. s, $CONH_2$); 7.39-7.31 (5H, m, H Ph); 5.08 (1H, d, ^2J = 12.5) and 4.99 (1H, d, ^2J = 12.5, $PhCH_2$); 3.04 (1H, dd, ^3J = 5.2, ^3J = 3.3, 2-CH); 2.39 (1H, dd, ^3J = 5.2, ^2J = 1.9, 3-CH_A); 2.33 (1H, dd, ^3J = 3.3, ^2J = 1.9, 3-CH_B). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 168.2; 160.8; 136.0; 128.4; 128.1; 127.9; 67.3; 35.7; 29.8. Found, %: C 60.05; H 5.42; N 12.83. $C_{11}H_{12}N_2O_3$. Calculated, %: C 59.99; H 5.49; N 12.72.

(+)-(2*R*)-*N*-Benzyloxycarbonylaziridine-2-carboxamide ((*R*)-5) was prepared from compound (*R*)-**4** in a similar manner to that described above. In the series consisting of six experiments isolated yields of isomer (*R*)-**5** varied from 80 to 90%. Mp 125-126°C. $[\alpha]_D^{20}$ 95° (*c* = 2.00, $CHCl_3$). Other analytical data of isomer (*R*)-**5** are identical to those of isomer (*S*)-**5**.

(-)-(2*S*)-Aziridine-2-carboxamide ((*S*)-1). A suspension consisting of isomer (*S*)-**5** (12.60 g, 57.5 mmol), MeOH (350 ml), THF (60 ml), and 10% Pd/C (1.26 g) was stirred under hydrogen atmosphere at ambient pressure and temperature for 40 min. The resulting mixture was filtered through Celite and evaporated under reduced pressure. The crude product (*S*)-**1** (4.90 g, 99%) was crystallized from abs. EtOH (16 ml) in the presence of activated carbon (0.24 g). Yield of pure compound (*S*)-**1** 4.09 g (83%). In the series consisting of seven experiments isolated yields of isomer (*S*)-**1** varied from 80 to 85%. Enantiomeric excess was determined by HPLC: τ_S 19.2 min, τ_R 21.2 min. Mp 153°C (measured by DSC) (mp 141-142°C [12]). $[\alpha]_D^{23}$ -63° (*c* = 5.50, MeOH). $[\alpha]_D^{23}$ -45° (*c* = 3.00, DMF) ($[\alpha]_D^{23}$ -44.8° (*c* = 1.07, DMF) [12]). IR spectrum, ν, cm^{-1}: 3300, 2063, 1650, 1522, 1467, 1447, 1318, 1220, 1183. ^{1}H NMR spectrum (D_2O), δ, ppm (*J*, Hz): 2.60 (1H, dd, ^3J = 6.0, ^3J = 3.1, 2-CH); 1.92 (1H, d, ^3J = 6.0, 3-CH_A), 1.85 (1H, d, ^3J = 3.1, 3-CH_B). ^{13}C NMR spectrum (D_2O+ MeOH), δ, ppm: 175.8; 29.3; 25.4. Found, %: C 41.96; H 7.03; N 32.68. $C_3H_6N_2O$. Calculated, %: C 41.85; H 7.02; N 32.54.

(+)-(2*R*)-Aziridine-2-carboxamide ((*R*)-1) was prepared from compound (*R*)-**5** in a similar manner to that described above. In the series consisting of five experiments isolated yields of isomer (*R*)-**1** varied from 80 to 85%. Mp 153°C (measured by DSC). $[\alpha]_D^{23}$ +63° (*c* = 5.5, MeOH). Other analytical data of isomer (+)-(*R*)-**1** are identical to those of isomer (*S*)-**1**

TABLE 1. Crystallographic Data for the Investigated Crystals of Isomer (*R*)-**1** and Racemate (±)-**1**

Parameters	(*R*)-**1**	(±)-**1**
Molecular formula	$C_3H_6N_2O$	$C_3H_6N_2O$
M	86.094	86.094
Crystal habit	Prism	Prism
Crystal size, mm	0.17 × 0.28 × 0.33	0.21 × 0.36 × 0.37
Crystal color	Colorless	Colorless
Crystal system	Orthorhombic	Monoclinic
Unit cell dimensions		
a, Å	5.0991(4)	5.0932(2)
b, Å	8.2306(6)	8.1643(4)
c, Å	9.9681(7)	9.9526(4)
β, deg.	90.0	93.452(3)
V, Å^3	418.35(5)	413.10(3)
Space group	$P2_12_12_1$	$P2_1/c$
Z	4	4
F(000)	184	184
μ, mm^{-1}	0.105	0.106
$d_{calc.}$, g/cm^3	1.367	1.384
$2\theta_{max}$ for data, deg.	60.0	55.0
Index ranges	-7<*h*<7; -11<*k*<11, -13<*l*<14	-6<*h*<6; -9<*k*<10, -12<*l*<12
Reflection collected	1235	1677
Independent reflections (R_{int})	732 (0.030)	945 (0.020)
Reflections with $I > N\sigma(I)$	539 (N = 2)	796 (N = 3)
Final *R*-factor	0.0429	0.0360
wR index for all data (on F^2)	0.1055	0.0921
Number of refined parameters	79	79
$(\Delta/\sigma)_{max}$	0.007	0.005
$\Delta\rho_{max}$ / $\Delta\rho_{min}$, e·Å	0.170 / –0.169	0.341 / –0.353

X-ray Studies of (*R*)- and (±)-Leakadine. The crystal structures of the racemate (±)-**1** and (*R*)-**1** were established by X-ray diffraction analysis. A single crystal automatic diffractometer Bruker-Nonius KappaCCD (MoKα-radiation, λ 0.71073 Å, *T* 193(2) K) was used for data collection. The crystal structures were determined by the direct method [22] and refined by full matrix least squares using SHELXL97 and maXus program packages [23, 24]. All of the hydrogen atoms were located from difference synthesis of electron density distribution. The non-hydrogen atoms were refined anisotropically, and the hydrogen ones, isotropically. The main crystallographic data and structure refinement parameters are given in Table 1. For further details, see crystallographic data for isomer (*R*)-**1** and racemate (±)-**1** deposited with the Cambridge Crystallographic Data Center as Supplementary Publication (CCDC numbers 863993 and 863994, respectively).

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