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Chemical and biochemical transformations of 5-ethoxycarbonyl-5-phenyl-2-isoxazolines

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Abstract

The salts, 2-methyl-5,5-disubstituted 4,5-dihydroisoxazolium methylsulfates comprising various substituents at the C-3 carbon atom were subjected to transformations. The structure of applied compounds permitted to monitor the effect of this factor on the transformation course of the 2-isoxazoline ring. The nucleophilic addition of cyanide anion to the selected salts enabled the obtaining of a next heterocyclic system of changed physicochemical and biological properties in comparison to the starting 2-isoxazolines. The diastereoselective hydrolysis of the cyanide group in 2-isoxazolidines by the bacteria strain *Rhodococcus rhodochrous* PCM 909 leads to the obtaining of a racemic mixture of the *trans*-hydroxyacid. The introduction of new functional groups into the heterocyclic ring made these compounds attractive objects for further chemical and microbial transformations and to study their biological activity.

Keywords: 4,5-Dihydroisoxazolium salts; Biological activity; *Rhodococcus rhodochrous*

1. Introduction

A number of compounds comprising in their structure a 2-isoxazoline ring or its hydrogenated derivative are known to show biological activity [1–5]. These compounds are often an intermediate in the total syntheses of ergot alkaloids (*chanoclavine*, *lysergic acid*), prostaglandins, macrolides and antibiotics (*acivicine*, *sarkomicin*, *mitomycin*, *vermiculine*, *paliclavine*, *nicomycine*) [6–13]. Thus, in recent years there is a noticeable increase in the interest of the substituted 2-isoxazoline system in biochemical studies, leading to the obtaining of biologically active compounds. This work, is a continuation of studies on the substituted 2-isoxazoline system [4,5,14–21]. The purpose of this study is examination of the reactivity of selected 2-isoxazoline systems in various chemical and biochemical reactions resulting in a change of their physicochemical and biological properties.

Studies on the reactivity of 2-isoxazoline systems are performed by first obtaining from them 4,5-dihydroisoxazolium salts [22], which are much more susceptible towards various types of reactions in comparison with the initial 2-isoxazoline, due to an increase in electrophility of the C-3 carbon atom, especially when in this position is electroacceptor substituent. The problem of obtaining and transformation of 4,5-dihydroisoxazolium 2-alkyl salts was dealt with in the seventies of last century [23]. Studies of the reactivity of 2-methyl-1,4-dihydroisoxazolium methylsulfates in the presence of the cyanide anion presented by Zadrożna and Kornacka [17] is a broadly described problem. The aim of this study was the reactivity of 2-methyl-4,5-dihydroisoxazoline methylsulfates-salts of cycloadducts of aliphatic nitryle oxides and methyl and phenyl esters p-substituted cinnamic acids.

2. Results and discussion

In this work the salts, 5,5-disubstituted 2-methyl-4,5-dihydroisoxazolium methylsulfates comprising various substituents at the C-3 carbon atom (CH_3, H, C_6H_5) were subjected to transformations. The structure of applied compounds permitted to monitor the effect of this factor on the transformation course of the 2-isoxazoline ring.

The nucleophilic addition of cyanide anion to the selected salts enabled the obtaining of a new heterocyclic system of

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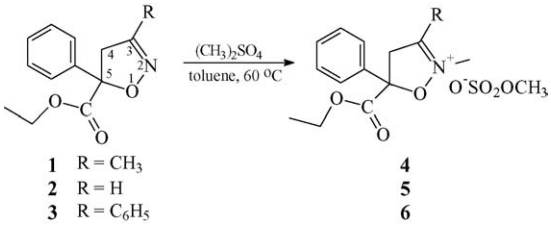
changed physicochemical and biological properties in comparison to the starting 2-isoxazolines. On the other hand, the introduction of new functional groups into the heterocyclic ring made these compounds attractive objects for further chemical and biochemical transformations and to study their biological activity.

2-Methyl-4,5-dihydroisoxazolium methylsulfates were obtained from the methylation with methylsulfate of 2-isoxazolines formed in the 1,3-dipolar cycloaddition of formyl-, acetyl-, and benzonitrile oxides with ethyl atropate (Scheme 1). Two standard methods of the 1,3-dipolar cycloaddition of nitrile oxides to α,β-unsaturated esters were utilized for the synthesis of the starting 2-isoxazolines, the method of Mukayama and Hoshino [24] for the synthesis of compounds **1** and **2** and that of Larsen and Torssell [25] for the synthesis of compound **3**, obtaining 5,5-disubstituted products.

The methylation reactions were carried out in toluene as solvent, with a fivefold excess of dimethyl sulfate with respect to the substrate used. The reaction mixture was maintained at 60–65 °C for 1 or 4 days depending on the structure of the substrate used (Scheme 1). The reaction yields were practically quantitative. The crude products, after evaporating of toluene, were subjected to further chemical transformations.

2.1. Cyanide anion nucleophilic addition

Studies on the reactivity of 2-methyl-4,5-dihydroisoxazolium methylsulfates in the presence of cyanide anion were carried out in a homogeneous phase system in an aprotic solvent DMSO and at various temperatures. The nucleophilic addition was carried out with the addition of a stoichiometric amount of the anion with respect to the starting



Scheme 1.

Table 1
Percentage share of the products of the 2-methyl-4,5-dihydroisoxazolium methylsulfate **4** reaction with a stoichiometric amount of sodium cyanide

Temperature	**7**-*trans* (%)	**7**-*cis* (%)	8 (%)	Yield (%)
room	34	66	–	67
60 °C	34	52	14	59
110 °C	22	7	71	44

2-isoxazoline. The reactions were carried out at room temperature as moderate reaction conditions, at 60 °C, i.e. the temperature of obtaining the salt and at 110 °C temperature of the heterocyclic ring cleavage, during 2 h, in DMSO.

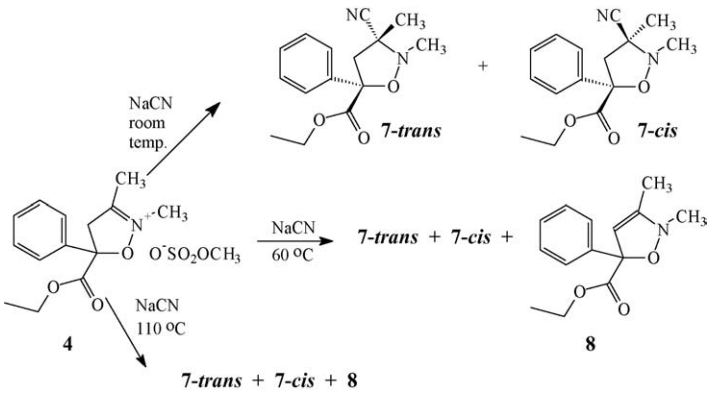
2-Methyl-4,5-dihydroisoxazolium methylsulfate **4**, obtained from compound **1**, was the first starting material used for these studies (Scheme 2).

In the reaction carried out at room temperature with a stoichiometric amount of sodium cyanide two diastereomeric addition products, **7**-*cis* and **7**-*trans*, were obtained, the structure of which was determined on the basis of an analysis of ^{1}H NMR spectra and ^{1}H NMR spectra with applying the *Overhauser nuclear effect* (NOE DIFF) (Scheme 2).

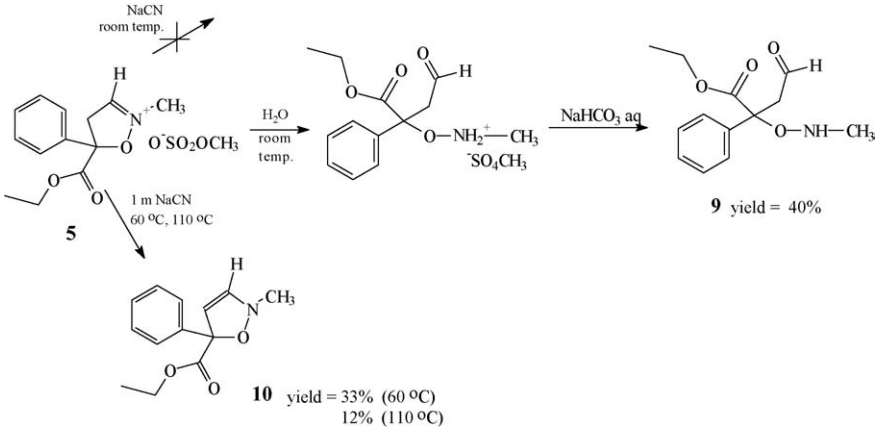
When carrying out the reaction at 60 °C a third compound **8** (3-isoxazoline derivative) appeared besides the mixture of products **7**. Identical three compounds, but with a different percentage share, were obtained during the reaction carried out at 110 °C (Table 1). A considerable increase in the percentage share of compound **8** took place at this temperature.

No product of the cyanide anion addition reaction was observed in the case of 4,5-dihydroisoxazolium salts bearing a proton (**5**) or phenyl group (**6**) at position 3 of the heterocyclic ring. In the case of the reaction of product **5**, obtained from 2-isoxazoline **2**, β-oxyaminoaldehyde **9** was isolated in a 40% yield, formed in the aqueous medium during processing of the post-reaction mixture [26]. However, during the reactions carried out at 60 and 110 °C only product **10** was obtained, probably resulting from the proton abstraction from position C-4 of the heterocyclic ring, where in this case the cyanide anion acted as a base (Scheme 3). Compound **11** and β-oxyaminophenylketone **12** were obtained during the reaction of 2-isoxazoline **6** (Scheme 4 and Table 2).

Compounds **7**–**12** were separated on a column chromatography (hexane/diethyl ether = 7:2).



Scheme 2.



Scheme 3.

2.2. Chemical reduction

Basing on the results of studies concerning the reduction of ester groups being substituents in 2-isoxazolines, the reduction of the mixture of compounds **7** was carried out with sodium borohydride to check whether it will be a sufficiently strong reducing agent to reduce the ester group also in this compound [21,27]. Unfortunately, attempts to reduce **7** with this reagent failed, even when used a 10-fold excess of $NaBH_4$ with respect to the applied substrate, prolonged the reaction time and raised the temperature. The unreacted ester was each time quantitatively isolated from the post-reaction mixture.

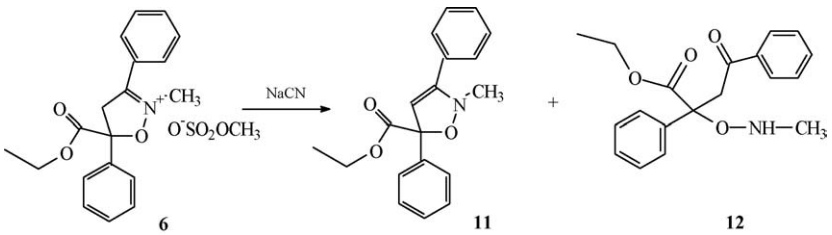
Therefore, the diastereoisomeric products **7** were subjected to the reduction with lithium aluminum hydride (LAH) to check the behavior of the ester and cyanide functional groups, presented at the heterocyclic ring. The reactions were carried out in anhydrous *d*iethyl ether at room temperature, in an argon atmosphere during 1.5 h. From the analysis of the products it was found that the reduction proceeded selectively with respect to the ester group. The reaction resulted in the obtaining of products in the form of racemic mixtures of diastereoisomeric alcohols **13** with a cyanide group at the C-3 position in an overall yield of 63% (Scheme 5). Prolongation of the reaction time caused a cleavage of the heterocyclic ring.

2.3. Microbial hydrolysis

According the literature [28–33], methods of hydrolysis of nitriles to corresponding amides or carboxylic acids can be divided into two groups, hydrolysis by isolated enzymes or hydrolysis by whole microorganisms. Kakey at al. used *Rhodococcus butanica* ATCC strain 21197 to carry out the stereoselective hydrolysis of 3-substituted glutaronitriles and obtained monoacids of S configuration [28,29]. Meth-Cohn and Wang [30–32] described a microbiological hydrolysis leading to carboxylic acids. The authors used *Rhodococcus rhodochrous* strain AJ270 and carried out the reaction in a phosphate buffer of pH 7.0 at 30 °C. With this approach, they were able to obtain heterocyclic compounds. In one of the latest works, Dadd et al. [33] reported regioselective transformation of nitriles, namely, hydrolysis of 2-, 3- and 4-(cyanomethyl)-benzonitriles by *R. rhodochrous* strain LL100-21.

In this report we describe microbiologically catalyzed selective hydrolysis of the cyanide group in the mixture of enantiomers of compound **7**. Two strains of the *Rhodococcus species NCIB 10554* (Institute of Biotransformation and Antibiotics, Warsaw) and *R. rhodochrous PCM 909* (Central Collection of Microorganisms, Institute of Immunology and Experimental Therapy of Polish Academy of Science, Wrocław) were used. Despite the literature reporting easy, selective hydrolysis of the cyanide group present in compounds bearing other constituents capable of biotransformation [28–33], in compounds **7**-*cis* and **7**-*trans* the ester group but the cyanide group underwent microbiological hydrolysis when *Rhodococcus species NCIB 10554* was applied. As a result of biotransformation the mixture of diastereoisomeric acids **14** (*trans*:*cis* = 65:35) was obtained (Scheme 6). Prolongation of the process from 48 h to 14 days resulted in the increase of the yield of reaction (to 14%).

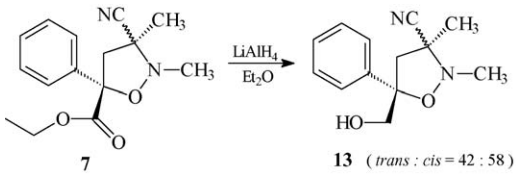
R. rhodochrous PCM 909 was applied for the hydrolysis of the mixture of diastereoisomeric alcohols **13**-*cis* and



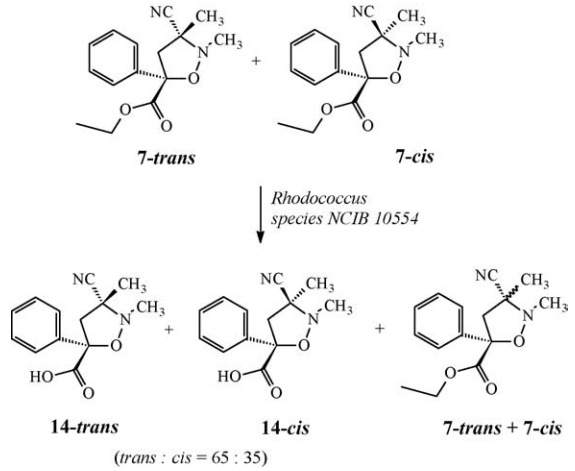
Scheme 4.

Table 2
Percentage share of the products of the 2-methyl-4,5-dihydroisoxazolium methylsulfate **6** reaction with a stoichiometric amount of sodium cyanide

Temperature	11 (%)	12 (%)	Yield (%)
Room	54	46	27
60 °C	88	12	6



Scheme 5.

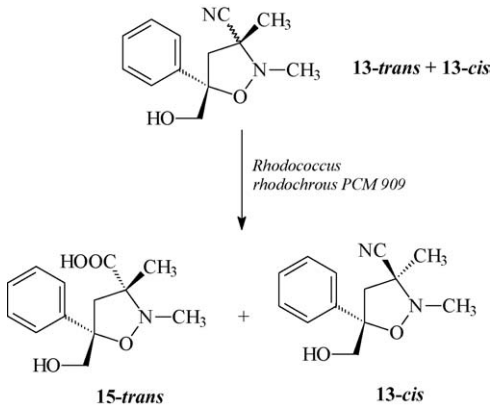


Scheme 6.

13-*trans* obtained by the reduction with LAH of compounds 7. The reaction catalyzed by this strain proceeded diastereoselectively during 48 h A racemic mixture of the hydroxyacid, *trans*-3-carboxy-2,3-dimethyl-5-hydroxymethyl-5-phenylisoxazolidine **15** was obtained with an 11% yield (Scheme 7).

2.4. Biological activity

All the compounds described here were examined for their biological activity. Strains of various cell wall structures were chosen for the tests: Gram-positive bacteria *Staphylococcus aureus* ATTC 6538, Gram-negative bacteria *Eschericha coli* ATTC 8739 and *Pseudomonas aeruginosa* ATTC 15442, and yeast *Candida albicans* ATTC 10231.



Scheme 7.

The biological activity of the tested compounds was tested by cylinder-plate method [5,34]. Briefly, Petri plates containing 15 ml of Muller–Hinton Agar were inoculated with a respective strain. The aliquot 100 μl of each tested solution of 20 mM stock was added to the cylinder placed on the surface of agar. Plates were incubated at 37 °C for 18 h for bacteria, and at 30 °C for 24 h for yeast, respectively. Growth inhibition was visualized as a clear region around the cylinder containing the studied compound. Diameter of the inhibition region was taken as a measure of growth inhibition.

Tetracycline (1 mM) and miconazole (1 mM) were used as a positive control for bacteria and fungi, respectively. The following inhibition regions were obtained: tetracycline-*E coli* = 19 mm, *S. aureus* = 27 mm, *Ps. aeruginosa* = 15 mm; miconazole, *C. albicans* = 23 mm.

Of 15 compounds tested in this study, only compounds **3**, **7**, **13** and **14** considerably inhibited growth of *C. albicans*. Respective inhibition regions were as follows: 11 mm, 10 mm, 13 mm and 12 mm.

3. Conclusions

In the reaction of various anions with the 2-methyl-4,5-dihydroisoxazolium salt, the product of the nucleophilic addition to the double bond or a 3-isoxazoline derivative is formed. The structure, percentage share and yield of products depend on the structure of the selected salt as well as on the reaction temperature.

Chemical reduction of the ester group present at the 2-isoxazoline ring, while preserving the heterocyclic system, to a corresponding primary alcohol by means of sodium borohydride is possible. The use of a stronger reducing agent, e.g. lithium aluminum hydride, is required in the case of isoxazolidines bearing an ester group.

The diastereoselective hydrolysis of the cyanide group in isoxazolidines **7** by means the bacteria strain *R. rhodochrous* PCM 909 leads to a racemic mixture of the hydroxyacid -*trans*-3-carboxy-2,3-dimethyl-5-hydroxymethyl-5-phenylisoxazolidine **15**.

Studies of the biological activity of the compounds obtained in this work encourage to continue the search of more effective derivatives in the isoxazole group influencing the growth inhibition of microorganisms.

4. Experimental

4.1. General

IR spectra were measured by means of a Specord 71 IR spectrophotometer, the band positions are expressed in cm^{-1}.

^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer in chloroform-d. The chemical shift values are expressed in δ ppm. Flash column chromatography was carried out using Merck Kieselgel 60 (230–400 mesh ASTM). The reactions were controlled by TLC. Elemental analysis was performed on a Perkin–Elmer CHNO analyzer.

4.2. General procedure for the preparation of 2-methyl-4,5-dihydroisoxazolium methylsulfates

2-Isoxazoline, fivefold excess of *d*imethyl sulfate and a small volume of toluene were placed in a dry flask. The content of the flask was heated for 1 or 4 days at 60 °C. After cooling this amount of toluene was added to the mixture and fell into layers. The upper toluene layer was decanted, and the lower one of the salt (thick, dark-brown oil) was washed with small portions of toluene. The remaining solvent was then removed under reduced pressure. The thus prepared salt was subjected to nucleophilic addition.

4.3. General procedure for carrying out the reaction of 2-methyl-4,5-dihydroisoxazolium methylsulfates with the cyanide anion

To a solution of 2-methyl-4,5-dihydroisoxazolium methylsulfate dissolved in DMSO (the solvent was distilled from above calcium hydride) sodium cyanide was added in a stoichiometric amount with respect to the initial 2-isozoxazoline. The reactions were carried out for 2 h at different temperatures: room temperature, 60 and 110 °C.

After reaction completion the mixture was poured into water; turbidity of the solution was noticed. The products were then extracted with diethyl ether. The extracts were dried with magnesium sulfate, and then ether was removed under reduced pressure and a mixture of crude products was obtained. The products obtained were purified by dissolution in hot hexane and boiling with active carbon, which was then filtered off on a fluted filter paper. After concentration the residue was chromatographed (hexane/diethyl ether = 7:2) to give products.

*4.3.1. Reactions of the 2,3-dimethyl-5-ethoxycarbonyl-5-phenyl-4,5-dihydroisoxazolium methylsulfate (**4**) with sodium cyanide*

Products, yellow oils; room temperature: yield: 67%, **7**-*trans*:**7**-*cis* = 34%:66%

60 °C: yield: 59%, **7-*trans***:**7-*cis***: **8** = 34%:52%:14%;

110 °C: yield: 44%, **7-*trans***:**7-*cis***: **8** = 22%:7%:71%.

*4.3.1.1. trans 2,3-Dimethyl-3-cyano-5-ethoxycarbonyl-5-phenylisoxazolidine (**7**-trans).* ^{1}H NMR: 1.20 (t, 3H, CH_3, J = 7 Hz), 1.55 (s, 3H, CH_3), 2.90 and 3.50 (2 × d, 2H, CH_2, J = 13.2 Hz), 2.89 (s, 3H, CH_3–N), 4.20 (q, 2H, CH_2, J = 7 Hz), 7.31–7.55 (m, 5H, Ar); ^{13}C NMR: 13.98 ($\underline{C}H_3CH_2$), 21.86 (CH_3), 39.11 (CH_3–N), 52.98 (C-4), 61.92 (C-3), 63.97 ($CH_3\underline{C}H_2$), 84.35 (C-5), 118.75 (C≡N), 124.93, 127.06, 128.47, 141.01 (Ar), 169,79 (C=O); IR (film) cm^{-1}: 2232 (C≡N),1732 (C=O).

*4.3.1.2. cis-2,3-Dimethyl-3-cyano-5-ethoxycarbonyl-5-phenylisoxazolidine (**7**-cis).* ^{1}H NMR: 1.21 (t, 3H, CH_3, J = 7 Hz), 1.46 (s, 3H, CH_3), 2,63 and 3,82 (2 × d, 2H, CH_2, J = 13.2 Hz), 2.88 (s, 3H, CH_3–N), 4.21 (q, 2H, CH_2, J = 7 Hz), 7.31–7.55 (m, 5H, Ar); ^{13}C NMR: 13.84 ($\underline{C}H_3CH_2$), 20.81 (CH_3), 38.93 (CH_3–N), 53.66 (C-4), 62.22 (C-3), 63.79 ($CH_3\underline{C}H_2$), 83.92 (C-5), 117.81 (C≡N), 124.49, 125.12, 128.33, 137.35 (Ar), 172.46 (C=O); IR (film) cm^{-1}: 2232 (C≡N),1732 (C=O).

4.3.1.3. Analysis of mixture of compounds. **7**-*trans* and **7**-*cis* $C_{15}H_{18}N_2O_3$ Calc.: C 65.69, N 10.22, H 6.57, Found: C 65.72, N 10.23, H 6.53.

4.3.1.4. 2,3-Dimethyl-5-ethoxycarbonyl-5-phenyl-3-isoxazoline (8). ^{1}H NMR: 1.37 (t, 3H, CH_3, J = 7.4 Hz), 2.34 (s, 3H, CH_3), 2.91 (s, 3H, CH_3-N), 4.23 (q, 2H, CH_2, J = 7.2 Hz), 6.63 (s, 1H, CH), 7.31–7.55 (m, 5H, Ar); ^{13}C NMR: 13.93 ($\underline{C}H_3CH_2$), 21.99 (CH_3), 39.06 (CH_3–N), 62.07 ($CH_3\underline{C}H_2$), 94.39 (C-5), 103.10 (C-4), 126.97, 127.50, 128.99, 144.82 (C-3), 146.17 (Ar), 175,90 (C=O); IR(film) cm^{-1}: 1731 (C=O); Analysis of compound $C_{14}H_{17}NO_3$ Calc.: C 68.02, N 5.67, H 6.88, Found: C 67.99, N 5.65, H 6.83.

*4.3.2. Reactions of the 5-ethoxycarbonyl-2-methyl-5-phenyl-4,5-dihydroisoxazolium methylsulfate (**5**) with sodium cyanide*

Products, yellow oils; room temperature: **9** yield: 40%; 60 °C: **10** yield: 33%; 110 °C: **10** yield: 12%.

*4.3.2.1. Ethyl 2-(N-methylamineoxy)-4-oxo-2-phenylbutaneate (**9**).* ^{1}H NMR: 1.43 (t, 3H, CH_3, J = 7.0 Hz), 2.67 (s, 1H, NH), 2.95 (s, 3H, CH_3–N), 3.08 and 3.89 (2 × d, 2H, CH_2, J = 13.5 Hz), 4.32 (q, 2H, CH_2, J = 7.0 Hz), 7.35–7.41 (m, 5H, Ar), 9.95 (m, 1H, CHO); ^{13}C NMR: 13.63 ($\underline{C}H_3CH_2$), 34.23 (CH_3–N), 47.67 (CH_2), 60.88 ($CH_3\underline{C}H_2$), 81.41 (C^{IV}), 127.37 (p-Ar), 128.92 (m-Ar), 131.11 (o-Ar), 135.29 (Ar), 174.06 (C=O), 200.65 (CHO); IR(film) cm^{-1}: 3432 (ν N–H), 2856 (CHO), 1734 (C=O), 1724 (C=O ald), 1528 (δ N–H); Analysis of compound $C_{12}H_{17}NO_4$ Calc.: C 6.25, N 5.86, H 7.11, Found: C 60.31, N 5.84, H 7.08.

*4.3.2.2. 5-Ethoxycarbonyl-2-methyl-5-phenyl-3-isoxazoline (**10**).* ^{1}H NMR: 1.21 (t, 3H, CH_3, J = 7.0 Hz), 2.67 (s, 3H, CH_3–N), 3.47 (q, 2H, CH_2, J = 7.0 Hz), 4.73 (d, 1H, CH-4, J = 3.6 Hz), 5.81 (d, 1H, CH-3, J = 3.6 Hz), 7.35–7.39 (m, 5H, Ar); ^{13}C NMR: 14.18 ($\underline{C}H_3CH_2$), 39.78 (CH_3–N), 61.62 ($CH_3\underline{C}H_2$), 96.97 (C-5), 109.55 (C-4), 127.82 (o-Ar), 131.21 (m-Ar), 132.68 (p-Ar), 136.28 (Ar), 141.08 (C-3), 175.09 (C=O); IR(film) cm^{-1}: 1728 (C=O); Analysis of compound $C_{12}H_{15}NO_3$ Calc.: C 65.16, N 6.33, H 6.79, Found: C 65.18, N 6.32, H 6.81.

*4.3.3. Reactions of the 3,5-diphenyl-5-ethoxycarbonyl-2-methyl-4,5-dihydroisoxazolium methylsulfate (**6**) with sodium cyanide*

Products, yellow oils; room temperature: yield: 27%, **11**:**12** = 54%:46%; 60 °C: yield: 6%, **11**:**12** = 88%:22%.

*4.3.3.1. 3,5-Diphenyl-5-ethoxycarbonyl-2-methyl-3-isoxazoline (**11**).* ^{1}H NMR: 1.26 (t, 3H, CH_3, J = 7.2 Hz), 2.98 (s, 3H, CH_3–N), 4.25 (q, 2H, CH_2, J = 7.2 Hz), 5.62 (s, 1H, CH), 7.32–7.51 (m, 10H, Ar); ^{13}C NMR: 14.04 ($\underline{C}H_3CH_2$), 37.28 (CH_3–N), 59.62 ($CH_3\underline{C}H_2$), 96.06 (C-5), 98.85 (C-4), 127.32 – 136.25 (Ar), 148.68 (C-3), 177.17 (C=O); IR(film) cm^{-1}: 1740 (C=O) Analysis of compound $C_{19}H_{19}NO_3$ Calc.: C 73.79, N 4.53, H 6.15, Found: C 73.77, N 4.55, H 6.14.

*4.3.3.2. Ethyl 2,4-diphenyl-2-(N-methylamineoxy)-4-oxobutaneate (**12**).* ^{1}H NMR: 1.45 (t, 3H, CH_3, J = 7.0 Hz), 2.63 (s, 1H, NH), 2.94 (s, 3H, CH_3–N), 2.99 and 3.87 (2 × d, 2H, CH_2, J = 13.2 Hz), 4.39 (q, 2H, CH_2, J = 7.0 Hz), 7.30–7.38 (m, 10H, Ar); ^{13}C NMR: 13.62 ($\underline{C}H_3CH_2$), 34.26 (CH_3–N), 46.50 (CH_2), 60.11 ($CH_3\underline{C}H_2$), 89.45 (C^{IV}), 126.23 (Ar), 126.98 (Ar); 127.37 (Ar), 128.92 (Ar), 129.11(Ar), 131.11 (Ar), 135.29 (Ar), 135.86 (Ar), 174.33 (C=O) 197.46 (C=O); IR(film) cm^{-1}: 3430 (ν N–H), 1732 (C=O),1528 (δ N–H); Analysis of compound $C_{19}H_{21}NO_4$ Calc.: C 69.72, N 4.28, H 6.42, Found: C 69.73, N 4.26, H 6.41.

*4.3.4. Reduction of the trans and cis 3-cyano-2,3-dimethyl-5-ethoxycarbonyl-5-phenylisoxazolidine (**7**) with LAH*

0.09 g (0.33 mmol) of the mixture of **7**-*cis* and **7**-*trans* in 20 ml of dry ethyl ether was added to the suspension of 0.07 g (2 mmol) of $LiAlH_4$ in 10 ml of ether at 5 °C in 5 min. After 90 min reflux to the stirred mixture was added slowly 10 ml of water and the product was extracted with ethyl ether. The extracts were dried with magnesium sulfate and ether was removed under reduced pressure.

Product, yellow oil, yield 0.048 g (63%); **13**-*trans*: **13**-*cis* = 42%: 58%.

*4.3.4.1. trans 3-Cyano-2,3-dimethyl-5-hydroxymethyl-5-phenylisoxazolidine (**13**-trans).* ^{1}H NMR: 1.57 (s, 3H, CH_3), 2.89 and 3.47 (2 × d, 2H, CH_2, J = 12.6 Hz), 2.87 (s, 3H, CH_3–N), 3.81 (s, 2H, CH_2), 7.28–7.36 (m, 5H, Ar); ^{13}C NMR: 21.32 (CH_3), 39.01 (CH_3–N), 50.96 (C-4), 64.03 (C-3), 70.51 (CH_2), 83.43 (C-5), 118.47 (C≡N), 127.48, 128.04, 128.57, 140.22 (Ar); IR (film) cm^{-1}: 3372 (OH), 2232 (C≡N).

*4.3.4.2. Cis-3-Cyano-2,3-dimethyl-5-hydroxymethyl-5-phenylisoxazolidine (**13**-cis).* ^{1}H NMR: 1.47 (s, 3H, CH_3), 2.56 and 3.70 (2 × d, 2H, CH_2, J = 12.6 Hz), 2.97 (s, 3H, CH_3–N), 3.79 (s, 2H, CH_2), 7.28–7.36 (m, 5H, Ar); ^{13}C NMR: 21.93 (CH_3), 38.83 (CH_3–N), 51.24 (C-4), 62.36 (C-3), 67.52 (CH_2), 84.74 (C-5), 118.99 (C≡N), 124.52, 125.10, 125.50, 143.35 (Ar); IR (film) cm^{-1}: 3372 (OH), 2232 (C≡N).

4.3.4.3. Analysis of mixture of compounds. **13**-*trans* and **13**-*cis* $C_{13}H_{16}N_2O_2$ calc.: C 67.24, N 12.07H 6.90, Found: C 67.23, N 12.06, H 6.87.

4.4. General procedures of biotransformation with Rhodococcus

Culture of *Rhodococcus* from an agar slop with solid medium (1.5% pancreatic digest of casein, 0.5% papaic digest of soya bean, 0.5% NaCl, 2% glucose, 1,5% agar, pH 7.0 ± 0.2) was used to inoculate 100 ml of liquid medium (1.7% pancreatic digest of casein, 0.3% papaic digest of soya bean, 0.5% NaCl, 2% glucose, 0.25% dipotassium hydrogen phosphate, pH 7.0 ± 0.2) in a 300 ml conical flask. Culture was incubated at 30 °C, in a rotary shaker at 120 rpm for 24 h. Than it was centrifuged at 4000 rpm for 30 min, and about 1 g of wet mass of *Rhodococcus* was resuspended in 50 ml of phosphate buffer solution (pH 7) in 250 ml flask. The culture was activated at 30 °C for 0.5 h with rotary shaking (120 rpm), and then 0.5 mmol of appropriate compound was added (dissolved in 1 ml of suitable solvent). The mixture was incubated at 30 °C with rotary shaking (120 rpm). After reaction completion the post-reaction mixture was extracted three times with 20 cm^3 of CH_2Cl_2. The solvent was then distilled off under reduced pressure. After preliminary analysis of IR and NMR spectra it was found that the ester and water are present in the reaction product. The product was dissolved in 5 cm^3 of methylene chloride and treated with 10 cm^3 of a saturated $NaHCO_3$ solution and thus the acid was converted into a sodium salt. The layers were separated and the organic layer was dried with magnesium sulfate, after which the solvent was distilled off under reduced pressure and the unreacted ester was obtained. The aqueous layer was neutralized with a diluted hydrochloric acid solution and also extracted with 20 cm^3 of methylene chloride. After drying the solution with magnesium sulfate the solvent was distilled off under reduced pressure, and thus the product, i.e. the acid, was obtained.

4.4.1. Microbiological hydrolysis of 7-trans and 7-cis using Rhodococcus species NCIB 10554

Product, yellow oil; 14 days, yield: 14%, **14**-*trans*:**14**-*cis* = 65%:35%.

*4.4.1.1. trans 5-Carboxy-3-cyano-2,3-dimethyl-5-phenyl-isoxazolidine (**14**-trans).* ^{1}H NMR: 1.56 (s, 3H, CH_3), 2.92 (s, 3H, CH_3–N), 2.98 and 3.40 (2 × d, 2H, CH_2, J = 13.4 Hz), 7.35–7.48 (m, 5H, Ar), 9.18 (s, 1H, OH); ^{13}C NMR: 20.76 (CH_3), 38.42 (CH_3–N), 53.38 (C-4), 64.08 (C-3), 83.60 (C-5), 117.97 (C≡N), 124.83, 125.56, 128.63, 140.60 (Ar), 173.22 (C=O); IR(KBr) cm^{-1}: 3028 (OH), 2236 (C≡N), 1720 (C=O).

*4.4.1.2. cis 5-Carboxy-3-cyano-2,3-dimethyl-5--phenyl-isoxazolidine (**14**-cis).* ^{1}H NMR: 1.47 (s, 3H, CH_3), 2.70 and 3.77 (2 × d, 2H, CH_2, J = 13.4 Hz), 2.89 (s, 3H, CH_3–N), 7.35–7.48 (m, 5H, Ar), 8.25 (s, 1H, OH); ^{13}C NMR: 21.74 (CH_3), 38.99 (CH_3–N), 52.83 (C-4), 63.95 (C-3), 84.16 (C-5), 118.52 (C≡N), 125.233, 125.59, 128.55, 136.50 (Ar), 176.23 (C=O); IR(KBr) cm^{-1}: 3028 (OH), 2236 (C≡N), 1720 (C=O).

Analysis of mixture of compounds **14**-*trans* and **14**-*cis* $C_{13}H_{14}N_2O_3$ Calc.: C 63. 42, N 11.38, H 5.69, Found: C 63.43, N 11.37, H 5.69.

4.4.2. Microbiological hydrolysis of 13-trans and 13-cis using R. rhodochrous PCM 909

Product, yellow oil; 48 h; yield: 0.014 g (11%).

*4.4.2.1. trans 3-Carboxy-2,3-dimethyl-5-hydroxymethyl-5-phenylisoxazolidine (**15**-trans).* ^{1}H NMR: 1.59 (s, 3H, CH_3), 3.00 and 3.45 (2 × d, 2H, CH_2, J = 13.4 Hz), 2.96 (s, 3H, CH_3–N), 3.88 (s, 2H, CH_2), 7.39–7.51 (m, 5H, Ar); ^{13}C NMR: 21.18 (CH_3), 38.77 (CH_3–N), 51.28 (C-4), 71.59 (CH_2), 77.92 (C-3), 89.96 (C-5), 125.57, 128.08, 128.91, 139.32 (Ar), 179.51 (C=O); IR(film) cm^{-1}: 3340 (OH), 1708 (C=O); Analysis of compound $C_{13}H_{16}NO_4$ Calc.: C 62.15, N 5.58, H 6.77, Found: C 62.20, N 5.57, H 6.63.

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