Katholieke *Universiteit* Nijmegen

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Enzymatic desymmetrization of prochiral dinitriles

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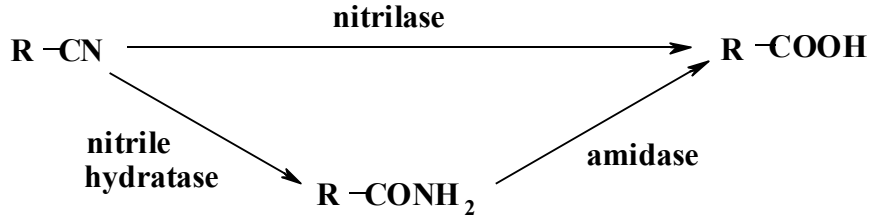
1. Introduction

Nitriles are very interesting compounds to organic chemists. These compounds are relatively easy to synthesize by nucleophilic substitution of a leaving group with cyanides, or by addition of cyanide derivatives to unsaturated bonds[1]. Nitriles are good by-products for further conversions.

The first step of the conversion is a hydrolysis of nitrile to either amide or acid. These reactions need a use of harsh conditions – very concentrated acids or bases; or heavy metal salts. Very high temperature is often required. Due to these facts only a limitted number of compounds may be synthesized via nitriles. Thus, it is not possible with sensitive compounds, and also in industrial scale – due to production of high amounts of salts, or very toxic waste[2,3,4].

A solution for these problems seems to be the use of nitrile hydrolysing enzymes. These enzymes work in mild conditions (in buffers with pH about neutral, and at mild temperatures). Moreover – these enzymes often present high regio- and stereoselectivity. Due to their properties they may be used for many syntheses of organic compounds (e.g. enantiopure); also those used in industry, or in pharmacy.



Scheme 1. Enzymatic ways of nitrile hydrolysis.

There are 2 enzymatic ways of nitrile hydrolysis[5] (scheme 1).

The first one is a direct hydrolysis of a nitrile to the acid catalysed by a nitrilase, and the second one consists of 2 steps. The first is a hydrolysis of a nitrile to the amide catalysed by a nitrile hydratase (NHase), and the second step is a hydrolysis of the amide to the acid catalysed by an amidase. Each of these enzymes may have its own specificity.

Nitrile hydrolysing enzymes may be used in industrial scale syntheses, in removal of waste, or in syntheses of drugs and other bioactive compounds. These enzymes are applied in the industrial production of acrylamide from acrylonitrile (by the Mitshubishi Rayon Co., and by Nitto Chemical, Japan)[6,7,8,9,10], in the synthesis of nicotinamide from nicotinonitrile (by Lonza AG, Switzerland)[10,11], and in the synthesis of 5-cyanovaleramide (intermediate in the production of some herbicides) from adiponitrile (by Du Pont, USA)[10].

Nitrile hydrolysing enzymes are relatively still unexplored, and they are very interesting objects of investigations for biochemists and organic chemists. They are very useful tools in organic syntheses.

The aim of this work was to do some investigations of enzymatic hydrolyses of substituted malonodinitriles; and also to study the mechanism of racemisation of 3-subsituted-

4-cyanobutyramides in presence of nitrile hydratase.

1.1. General experimental section

The *Rhodococcus erythropolis* NCIB 11450 strain and the cell-free extract isolated from this organism (containing NHase, but no amidase activity) were provided by Prof. Helmut Schwab Group, Technical University of Graz, Austria.

All NMR measurements were performed on Bruker WM-200 and Bruker AM-300 machines.

GC's were performed on HP 5890 (carrier gas N_2) on HP-1 (25m x 0.32mm x 0.17m) column.

GC-MS was performed on the double focusing VG7070E spectrometer.

2. Investigations of enzymatic hydrolyses of substituted malonodinitriles

2 types of stereospecific enzymatic hydrolyses of nitriles may be distinguished. The first one is a hydrolysis of a racemic mixture of nitriles (resolution). The idea here is to hydrolyse both enantiomers with different velocities, and a formation of one product from one of isomers is favoured. This type is the most common one; there were a lot of reactions of this kind investigated. A disadvantage of this type of hydrolysis is 50% maximal yield.

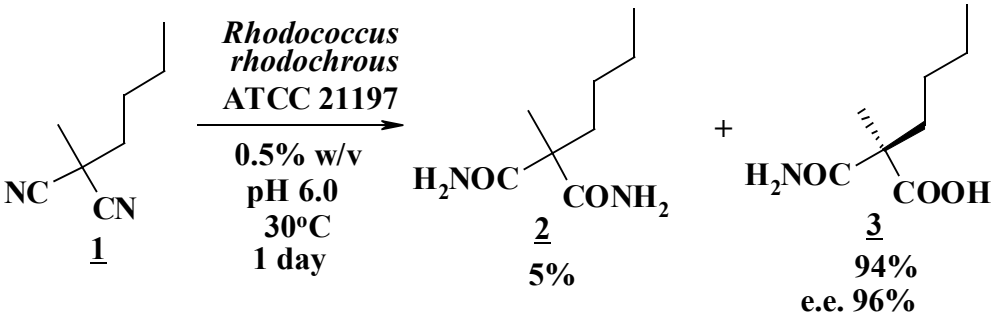
The second type of enzymatic hydrolysis of nitriles is an asymmetric hydrolysis of prochiral dinitriles. The idea here is the preferential hydrolysis of one of the nitrile groups, which leads to an enantiometrically enriched product. A yield here may be max. 100%; however, there is no sigificant evidence of this type of reactions.

Quite a few investigations with 3-substituted glutarodinitriles have been made, and only a few with differently substituted malonodinitriles.

Some chemistry of these compounds is studied in this chapter.

2.1. Investigations of 2,2-dicyanohexane as a substrate

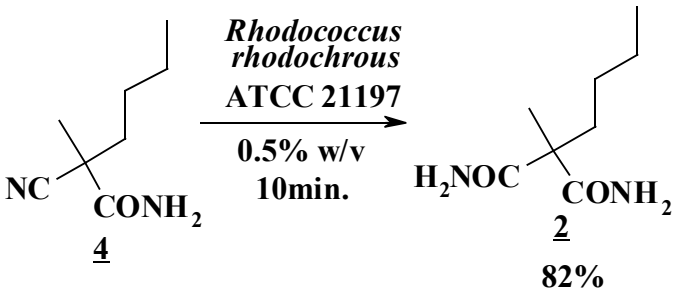
Yokoyama et al.[12] reported the microbial hydrolysis of 2,2-dicyanohexane (**1**) with *Rhodococcus rhodochrous* ATCC 21197 (scheme 2). They obtained 5% of 2-(*n*-butyl)-2-methylmalonodiamide (**2**) and (R)-2-(*n*-butyl)-2-methylmalonoamide (**3**) with 94% yield and 96% e.e.



Scheme 2. Asymmetric hydrolysis of 2,2-dicyanohexane with *R. rhodochrous* ATCC 21197.

Used *R. rhodochrous* strain had a very fast non selective NHase, and a slow (R)-selective amidase. Non-selectivity of the NHase was proven by the hydrolysis of racemic 2-cyano-2-methylhexanamide (**4**) (scheme 3). It yielded 82% of **2** without any recovery of the substrate. It means that only the amidase was responsible for high e.e. in the microbial hydrolysis of **1**.

It was very interesting to compare hydrolyses of **1** using different *Rhodococcus* strains – *rhodochrous* ATTC 21197 mentioned above, and *erythropolis* NCIB 11450 investigated here.



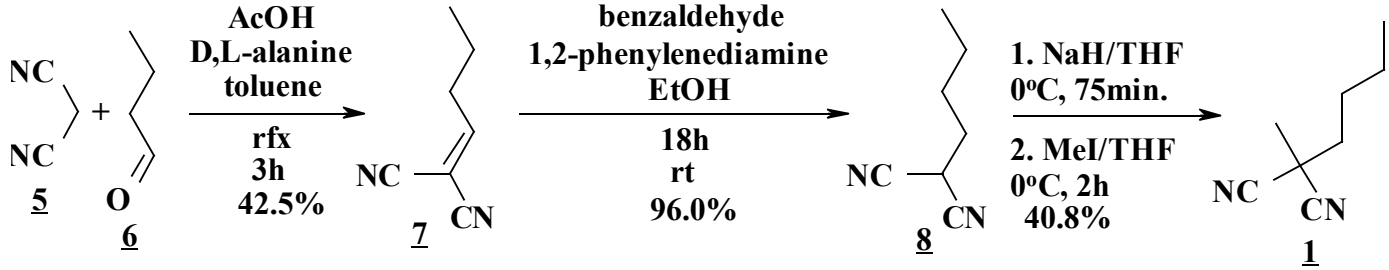
Scheme 3. Hydrolysis of racemic 2-cyano-2-methyl-hexanamide with *R. rhodochrous* ATCC 21197.

There was also the enzymatic hydrolysis of 2-cyano-2-methyl-3-phenylpropionitrile mentioned in the literature[13], but without any further details.

The aim of this part of work was: 1) to synthesise the substrate 2,2-dicyanohexane (**1**); 2) to perform the enzymatic hydrolysis of **1**, and to identify the product of this reaction; and – if the product was chiral - 3) to synthesise a racemic product for seeking an assay for determination of e.e., and further chemistry.

2.1.1. Synthesis of 2,2-dicyanohexane (1)

Synthesis of 2,2-dicyanohexane (**1**; scheme 4) was performed via the Knoevenagel condensation of malonodinitrile (**5**) with butyraldehyde (**6**), followed by the reduction of obtained 1,1-dicyanopent-1-ene (**7**) to 1,1-dicyanopentane (**8**), which was converted to **1** by the methylation.



Scheme 4. Synthesis of 2,2-dicyanohexane.

The first step was the Knoevenagel condensation of **5** and **6**, leading to **7** (2.1.1.1.). This reaction[14] underwent with relatively good yield (42.5%). The problem here was the fact that butyraldehyde gives some side reactions (e.g. oxidation), especially at higher temperatures. Similar procedures were applied to the syntheses of **15** (2.2.1.1.; excellent, higher than 81.6% yield was obtained due to higher activity of aromatic aldehydes vs aliphatic ones) and **17** (2.2.3.1.; 64% yield due to the similar reason).

The second step of this synthesis, the reduction of **7** to **8**, was much more difficult.

The first attempt (2.1.1.2.), using Mg/HCl for generation of hydrogen in situ[15], wasn't successful - it gave only 12.5% yield. It was performed similarly to the conversion of **15** to **13**

(2.2.1.2.), which gave also very small 5.45% yield; and to the reduction of **30** to **31** (2.3.2.2.). In contrary, the latter process gave excellent 93.0% yield.

The second attempt (2.1.1.3.) was reduction of **7** with $NaBH_4$[16]; but the yield here was even smaller (8.0%), than in the reaction with Mg/HCl. However; it was a good reagent for the reduction of **15** (2.2.1.3.); it gave much better, 81.6% yield. The similar procedure was also used for the reduction of **17** to **18** (2.2.3.4.), but the ester moiety was reduced giving primary alcohol **19** mainly.

The third attempt (2.1.1.4.) using benzaldehyde as a reducing agent, and 1,2-phenylenediamine (forms a reactive compound with benzaldehyde) was successful; it gave excellent 96.0%, yield. This procedure was also used for the conversions of **10** to **11** (2.1.3.2.), which gave 24.3% yield; **17** to **18** (2.2.3.3.); and also **27** to **26** (2.3.1.1.), which, in contrary, gave excellent 90% yield.

The third step of this synthesis was the methylation of **8** to **1** (2.1.1.5.). It was performed by the abstraction of the α–hydrogen atom (with NaH), and the substitution with the methyl group (from MeI). It gave 40.8% yield. It might be higher in a larger scale due to humidity, which decomposes more (in percentage) NaH in a smaller scale. The similar procedure was applied for the conversion of **11** into **12** (2.1.3.3.) with almost the same result - it gave 37.9% yield.

The whole synthesis gave 16.6% overall yield. It is not excellent, but enough to obtain some substrate for enzymatic reaction, **1**.

2.1.1.1. Synthesis of 1,1-dicyanopent-1-ene (7)

8.25g (125mmol) of malonodinitrile (**5**), 10.0ml (8.17g; 113mmol) of butyraldehyde (**6**), 332mg of D,L-alanine, 6.7ml of acetic acid and 334ml of toluene were stirred under a Dean-Stark trap in reflux conditions for 3h. The mixture was washed with 334ml of water. The organic layer was dried over Na_2SO_4, drained and evaporated. The crude product was purified on a silica gel column using heptane - ethyl acetate 4:1 (v/v) as an eluent. 5.77g (48.1mmol) of liquid 1,1-dicyanopent-1-ene (**7**) were obtained with 42.5% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 1.01 (3H, CH_3, t, 7.4Hz), 1.50-1.70 (2H, 4-CH_2, m), 2.57 (2H, 3-CH_2, m), 7.34 (1H, CH, t, 8.0Hz)

2.1.1.2. Synthesis of 1,1-dicyanopentane (8, reduction with Mg/HCl)

1.10g (9.17mmol) of 1,1-dicyanopent-1-ene (**7**), 13.4g (558mmol) of Mg and 147ml of methanol were stirred until first bubbles of gas had been formed (40min.). The mixture was cooled in an ice bath, and 254ml of 6M $HCl_{(aq)}$ were added dropwise during 250min; the reaction was kept in the ice bath with stirring. Then it was stirred for 19 hours more, and extracted 6 x 106ml of diethyl ether. The combined organic layers were extracted with 212ml of saturated $NaCl_{(aq)}$. The

organic layer was dried over $MgSO_4$, drained, and solvents were evaporated. The obtained mixture was purified on a silica gel column using heptane - ethyl acetate 9:1 (v/v) as an eluent. 140mg (1.15mmol) of colourless oil of 1,1-dicyanopentane (**8**) were obtained with 12.5% yield.

2.1.1.3. Synthesis of 1,1-dicyanopentane (8, reduction with $NaBH_4$)

736mg (6.13mmol) of 1,1-dicyanopent-1-ene (**7**) were dissolved in 28ml of ethanol, and cooled in an ice-salt bath. Then 403mg (10.7mmol) of $NaBH_4$ were added in portions. The reaction was stirred in the ice-salt bath for 90min., then 4ml of 6M $HCl_{(aq)}$ and 5ml of water were added. The mixture was extracted with 30ml and 3 x 20ml of diethyl ether. The combined organic layers were extracted with 20ml of 5% $NaOH_{(aq)}$. The organic layer was dried over $MgSO_4$, drained, and the solvent was evaporated. The obtained mixture was purified on a silica gel column using heptane - ethyl acetate 6:1 (v/v) as an eluent. 60mg (0.49mmol) of liquid 1,1-dicyanopentane (**8**) were obtained with 8.0% yield.

2.1.1.4. Synthesis of 1,1-dicyanopentane (8, reduction with benzaldehyde)

900mg (7,50mmol) of 1,1-dicyanopent-1-ene (**7**), 893mg (8.75mmol) of 1,2-phenylenediamine, 0.850ml (887mg; 8.37mmol) and 70ml of ethanol were stirred at room temperature for 18h. The solvent was evaporated; the obtained mixture was purified on a silica gel column using heptane - ethyl acetate 6:1 (v/v) as an eluent. 878mg (7.20mmol) of liquid 1,1-dicyanopentane (**8**) were obtained with 96.0% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.95 (3H, CH_3, t, 7.2Hz), 1.32-1.52 (2H, 4-CH_2, m), 1.53-1.69 (2H, 3-CH_2, m), 1.98-2.10 (2H, 2-CH_2, m), 3.73 (1H, CH, t, 6.9Hz)

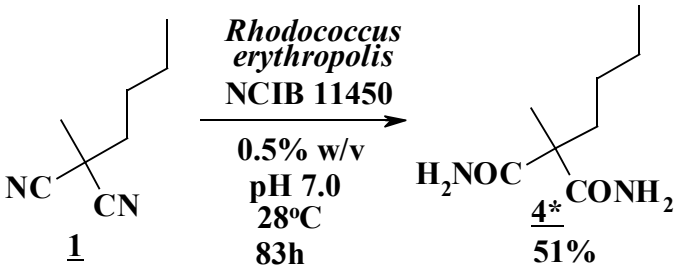
2.1.1.5. Synthesis of 2,2-dicyanohexane (1)

468mg (19.5mmol) of NaH were dissolved in 21ml of THF and cooled in an ice-salt bath. The solution of 1.87g (15.3mmol) of 1,1-dicyanopentane (**8**) in 21ml of THF was added dropwise during 45min. 10ml of THF were added, the mixture was stirred in the ice-salt bath during 30min. more, then the solution of 1.20ml (2,74 g; 19.3mmol) CH_3I in 21ml of THF was added dropwise. The reaction was kept in the ice-salt bath for 2h. Then it was quenched by addition of 60ml of water. The mixture was saturated with $(NH_4)_2SO_4$ and extracted 3 x 50ml of ethyl acetate. The organic layers were combined, dried over Na_2SO_4. After draining and evaporation of the solvent, the residue was purified on a silica gel column using heptane - ethyl acetate 8:1 (v/v) as an eluent. 848mg (6.24mmol) of liquid 2,2-dicyanohexane (**1**) were obtained with 40.8% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.97 (3H, 6-CH_3, t, 7.2Hz), 1.34-1.54 (2H, 5-CH_2, m), 1.55-1.73 (2H, 4-CH_2, m), 1.79 (3H, 1-CH_3, s), 1.87-1.98 (2H, 3-CH_2, m)

2.1.2. Enzymatic hydrolysis of 2,2-dicyanohexane (1) using *Rhodococcus erythropolis* NCIB 11450 cells

The enzymatic hydrolysis of **1** (scheme 5) was performed using *Rhodococcus erythropolis* NCIB 11450 cells (2.1.2.1.).



Scheme 5. Enzymatic hydrolysis of 2,2-dicyanohexane with *R. erythropolis* NCIB 11450.

The aim of this experiment was to identify the products of this reaction. The ^{1}H-NMR spectrum showed the presence of characteristic amide signals, and the GC/MS experiment confirmed the presence of the cyano-amide **4***. Similarly, cyano-amides were the products in the analogous hydrolyses of **13** (2.2.2.1.) and **26** (2.3.2.1.). It was confirmed, after the synthesis of **4** (2.1.3.), by TLC and GC.

4* was obtained with 51% yield after 83h comparing to 49% after 2h obtained in the hydrolysis of **13**. However, the yield might have been higher if the reaction had been performed in a larger scale, due to probable loss of compound, and errors in weighing. The time of conversion might have been shorter, either.

It's a very interesting fact that the *R. erythropolis* NCIB 11450 strain used here gave the cyano-amide **4*** as a product, whereas *R. rhodochrous* ATCC 21197 gave the amido-acid **3**[12].

The use of *R. erythropolis* NCIB 11450 strain gave smaller yields, and took more time; however, this reaction should be optimised in a larger scale as mentioned above.

The next goal was to synthesise **4**, the product of the enzymatic hydrolysis of **1**. It was necessary to find some assay to determine the e.e.s of this reaction, which would be also needed for the optimisation of this reaction. It was also a need to confirm identification of the product of the hydrolysis of **1**, and to prepare for some further chemistry of this compound.

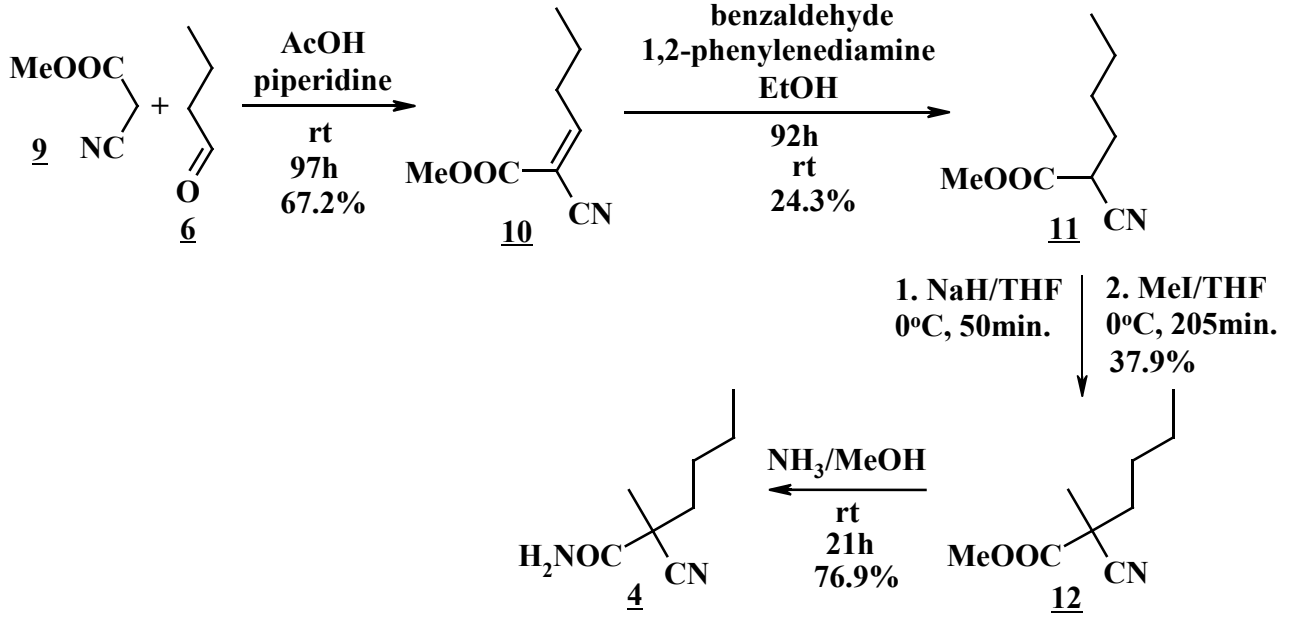
2.1.2.1. Enzymatic hydrolysis of 2,2-dicyanohexane (1) with *Rhodococcus erythropolis* NCIB 11450 cells

The mixture of 20μl (19mg; 0.14mmol) of 2,2-dicyanohexane (**1**) and 24mg of *Rhodococcus erythropolis* NCIB 11450 cells in 5.0ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken

250rpm in 28 °C for 83 hours. The mixture was saturated with $(NH_4)_2SO_4$, and extracted 5 x 5ml of ethyl acetate. Combined organic layers were dried over Na_2SO_4, drained, and the solvent was evaporated. The obtained mixture was purified on a silica gel column using heptane - ethyl acetate 8:1 (v/v) to elute non-reacted substrate, and then ethyl acetate to elute 11mg (0.071mmol; 51% yield) of product, which was identified (TLC, ^{1}H-NMR, GC, GC/MS) as 2-cyano-2-methylhexanamide (**4***).

2.1.2.3. Synthesis of 2-cyano-2-methylhexanamide (4)

The synthesis of **4** (scheme 6) was performed via the Knoevenagel condensation of methyl cyanoacetate (**9**) with **6** to give methyl (*E,Z*)-2-cyanohex-2-enoate (**10**), the subsequent reduction to methyl (*R,S*)-2-cyanohexanoate (**11**), the methylation to methyl (*R,S*)-2-cyano-2-methylhexanoate (**12**), and the final conversion to (*R,S*)-2-cyano-2-methylhexanamide (**4**).



Scheme 6. Synthesis of (*R,S*)-2-cyano-2-methylhexanamide.

The first step was the Knoevenagel condensation of **9** with **6** leading to **10**[17] (2.1.3.1.). It gave good 67.2%, yield. A mixture of *E* and *Z* isomers was formed (^{1}H-NMR); however, it did not play an important role in the further conversions. The similar procedure was used for the synthesis of **17** (2.2.3.2.), which gave much higher 84.3% yield of only one of the *E,Z* isomers, due to higher activity of aromatic aldehydes comparing to aliphatic ones.

The second step was the reduction of **10** to **11** (2.1.3.2.) using benzaldehyde/ 1,2-phenylenediamine. It gave surprisingly small (24.3%) yield comparing to the analogous reduction of **7** to **8** procedure (96.0%; 2.1.1.4.). The similar procedure was also applied in the synthesis of **18** (2.2.3.3.) and **26** (2.3.1.2.), where it gave excellent 90% yield.

The third step was the methylation of **11** to **12** (2.1.3.3.). It gave similar yield (37.9% vs 40.8%) to the analogous methylation of **8** to **1** procedure (2.1.1.5.).

The final step of the synthesis of **4** was the substitution of the active methoxy group of the ester moiety of **12** with the amino group of ammonia (2.1.3.4.). It gave very good 76.9% yield. The procedure used here was also applied in the syntheses of **16** (2.2.3.5.), **29** (2.3.2.3.; 40.2% yield), **37** (3.1.1.4.) and **37*** (3.1.2.3.).

The whole synthesis of **4** gave 4.76% yield, which may be optimised by the use of a different reduction procedure for the synthesis of **11**.

The synthesised product of enzymatic hydrolysis (2.1.2.2.1.), **4**, will be used for seeking a method of determining of e.e. of this reaction, and for potential further chemistry.

2.1.3.1. Synthesis of methyl (*E,Z*)-2-cyanohex-2-enoate (10)

5.06ml (4.05g; 56.2mmol) of butyraldehyde (**6**), 5.50ml (6.18g; 62.4mmol) of methyl cyanoacetate (**9**), 7.5ml of acetic acid and 0.2ml of piperidine were stirred at room temperature for 97h. 10ml of water were added, the mixture was extracted 3 x 20ml of dichloromethane. The combined organic layers were dried over Na_2SO_4. The oil obtained after draining and evaporation of the solvent was distilled under reduced pressure. 5.78g (37.8mmol) of oilish methyl 2-cyanohex-2-enoate (**10**; mixture of E and Z isomers) were obtained with 67.2% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.95 (3H, 6-CH_3, t, 7.5Hz), 1.19-1.73 (4H, 4- and 5-CH_2, m), 3.84 and 3.86 (3H, OCH_3, s), 7.37-7.72 (1H, CH, m)

2.1.3.2. Synthesis of methyl (*R,S*)-methyl 2-cyanohexanoate (11)

1.33g (8.69mmol) of methyl (E,Z)-2-cyanohex-2-enoate (**10**), 905mg (8.38mmol) of 1,2-phenylenediamine, 0.900ml (940mg; 8.86mmol) of benzaldehyde, 77ml of ethanol were stirred at room temperature for 92h. The residue obtained after evaporation of solvent was purified on a silica gel column using heptane - ethyl acetate 8:1 (v/v). 315mg (2.03mmol) of liquid methyl (*R,S*)-2-cyanohexanoate (**11**) were obtained with 24.3% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.92 (3H, 6-CH_3, t, 6.9Hz), 1.23-1.56 (4H, 4- and 5-CH_2, m), 1.87-2.00 (2H, 3-CH_2, m), 3.50 (1H, CH, t, 6.7 Hz), 3.80 (3H, OCH_3, s)

2.1.3.3. Synthesis of methyl (*R,S*)-2-cyano-2-methylhexanoate (12)

179mg (7.46mmol) of NaH were dissolved in 8.0ml of THF, the solution was cooled in an ice-salt bath. The solution of 1.02g (6.61mmol) of methyl (*R,S*)-2-cyanohexanoate (**11**) in 8.0ml of THF was added dropwise during 15 min. Another 8.0ml of THF were added; the mixture was stirred in the ice-salt bath for 35min more. Then the solution of 0.450ml (1.03g; 7.23mmol) of CH_3I in 8.0ml of THF was added dropwise during 8min. The mixture was stirred in the ice-salt bath for

3h and 25min and quenched with 20ml of water. The mixture was saturated with $(NH_4)_2SO_4$, and extracted. The water layer was extracted with 3 x 20ml of ethyl acetate. The combined organic layers were dried over $MgSO_4$. The residue obtained after draining and evaporation of solvent was purified on a silica gel column using heptane - ethyl acetate 8:1 (v/v) as an eluent. 423mg (2.50mmol) of liquid methyl (*R,S*)-2-cyano-2-methylhexanoate (**12**) were obtained with 37.9% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.91 (3H, 6-CH_3, t, 7.2Hz), 1.25-1.56 (4H, 4- and 5-CH_2, m), 1.59 (3H, 2-CH_3, s), 1.66-2.01 (2H, 3-CH_2, m), 3.82 (3H, OCH_3, s)

2.1.3.4. Synthesis of (*R,S*)-2-cyano-2-methylhexanamide (4)

118mg (0.698mmol) of methyl (*R,S*)-2-cyano-2-methylhexanoate (**12**) were dissolved in 32ml of 7M NH_3 in methanol. The solution was stirred in a stoppered flask for 21h 15min. The solvent was evaporated, the residue was purified on a silica gel column using heptane - ethyl acetate 1:1 (v/v), and then ethyl acetate as eluents. 82.7mg (0.537mmol) of white solid (*R,S*)-2-cyano-2-methylhexanamide (**4**) were obtained with 76.9% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 0.92 (3H, 6-CH_3, t, 7.0Hz), 1.24-1.47 (4H, 4- and 5-CH_2, m), 1.58 (3H, 2-CH_3, s), 1.80-2.10 (2H, 3-CH_2, m), 5.53 (1H, NH, bs), 6.28 (1H, NH, bs)

2.2. Investigations of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile as a substrate

2-cyano-3-(p-hydroxyphenyl)-propionitrile may be a very interesting subject of these investigations due to different reasons. It may be a very useful compound for further chemistry.

E.g. if the cyano-amide (i.e. 2-cyano-3-(*p*-hydroxyphenyl)-propionamide) was the product of an enzymatic hydrolysis, it might be converted into tyrosine. It might be a novel way of a synthesis of this amino acid and its derivatives. It might be also useful for syntheses of labelled tyrosines.

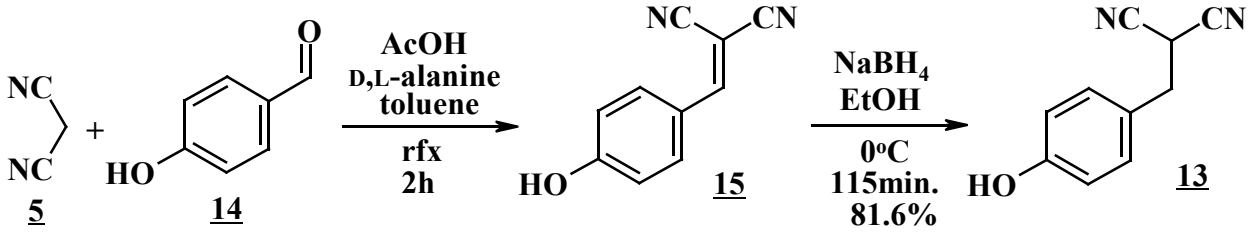
There is no literature data for a use of this substrate in enzymatic hydrolyses.

This part of work contains: 1) the synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile; 2) enzymatic hydrolyses of the substrate, and identification of their product; - if the product was chiral - 3) a synthesis of a racemic product; and - if it was the cyano-amide - 4) a conversion into tyrosine.

2.2.1. Synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13)

The synthesis of **13** was performed via the Knoevenagel condensation of malonodinitrile (**5**)

with *p*-hydroxybenzaldehyde (**14**) leading to 2-cyano-3-(*p*-hydroxyphenyl)-acrylonitrile (**15**), and the reduction to 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**; scheme 7).



Scheme 7. Synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile.

The first step of this synthesis was the Knoevenagel condensation of **5** and **14**[14] leading to **15** (2.2.1.1.). It gave excellent yield; however, it was not measured precisely, since the crude product was obtained (although the ^{1}H-NMR spectrum did not show any presence of impurities). A reason of the high yield might have been a fact, that the aromatic aldehyde had been condensed; and it had been much more active, than the aliphatic one comparing to the analogous procedure of synthesis of **7** (42.5% yield; 2.1.1.1). The synthesis of **17** (2.2.3.1.; 64% yield) was performed in the similar manner.

The subsequent step was the reduction of **15** to **13**. This again was the limiting step of this synthesis.

The first attempt was the reduction with Mg/HCl[15], but it gave a very small yield (5.45%; 2.2.1.2.); even smaller than in the similar reduction of **7** to **8** (12.5%; 2.1.1.2.). The same procedure was applied in the synthesis of **31** from **30** (2.3.2.2.), but here it gave much higher yield – 93.0%.

The second attempt was the reduction with $NaBH_4$[16] (2.2.1.3.). It gave very good 81.6% yield; whereas the analogous procedure used for the reduction of **7** to **8** gave only 8.0% yield (2.1.1.3.). It was also used for the conversion **17** to **18** (2.2.3.4.), but primary alcohol **19** was formed mainly.

The whole synthesis of the substrate for the enzymatic hydrolysis **13** gave very good 81.6% yield.

2.2.1.1. Synthesis of 2-cyano-3-(p-hydroxyphenyl)-acrylonitrile (15)

1.68g (12.7mmol) of *p*-hydroxybenzaldehyde (**14**), 1.03g (15.6mmol) of malonodinitrile (**5**), 41mg of D,L-alanine, 0.85ml of acetic acid and 41ml of toluene were stirred in reflux conditions under a Dean-Stark trap for 2h. 18ml of THF were added to dissolve precipitated crystals. The mixture was extracted 3 x 40ml of water. The organic layer was dried over Na_2SO_4. After it had been drained and solvents had been evaporated, 2.33g of yellow solid crude 2-cyano-3-(*p*-hydroxyphenyl)-acrylonitrile (**15**) were obtained.

^{1}H-NMR (200MHz, $CDCl_3$): δ 5.30 (1H, OH, s), 6.96 (2H, ArH, d, 8.8Hz), 7.65 (1H, CH, s), 7.88 (2H, ArH, d, 8.8Hz)

2.2.1.2. Synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13; reduction with Mg/HCl)

2.33g (12.7mmol) of crude 2-cyano-3-(*p*-hydroxyphenyl)-acrylonitrile (**15**), 13.3g (554mmol) of Mg and 141ml of methanol were stirred util first bubbles of gas had been formed (30min.). The mixture was cooled in an ice bath, and 260ml of 6M $HCl_{(aq)}$ solution were added dropwise during 4 hours; the mixture was stirred and kept in the ice bath. After whole HCl solution had been added, the reaction was kept at room temperature for next 20 hours. Then it was extracted 6 x 106ml of diethyl ether. The combined organic layers were extracted with 212ml of saturated $NaCl_{(aq)}$. The organic layer was dried over $MgSO_4$, drained, and solvents were evaporated. The crude mixture was purified on a silica gel column using ethyl acetate - heptane 1:1 (v/v) as an eluent. 119mg (0.692mmol) of solid 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**) were obtained with 5.45% yield calculated from *p*-hydroxybenzaldehyde (**14**).

2.2.1.3. Synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13, reduction with $NaBH_4$)

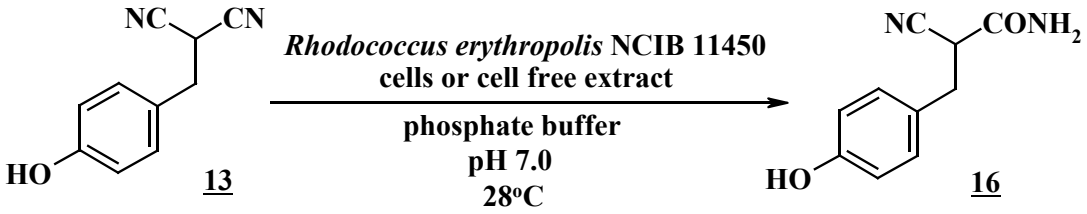
1.26g (7.43mmol) of crude 2-cyano-3-(*p*-hydroxyphenyl)-acrylonitrile (**15**) were dissolved in 26ml of ethanol. The solution was stirred and cooled in an ice-salt bath. 388mg (10.3mmol) of $NaBH_4$ were added during 15min. The reaction was carried for 100min. with stirring and cooling in the ice-salt bath, and then quenched with 2ml of conc. $HCl_{(aq)}$. 25ml of water were added, the solution was saturated with $(NH_4)_2SO_4$, and extracted 5 x 25ml of ethyl acetate. The combined organic layers were extracted with 25ml of 1M $NaOH_{(aq)}$. The organic layer was dried over MgSO4. The residue obtained after draining and evaporation of the solvent was purified on a silica gel column using ethyl acetate - heptane 1:1 (v/v) as an eluent. 1.05g of white solid of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**) were obtained with 81.6% yield.

^{1}H-NMR (200MHz, $CDCl_3$): δ 3.23 (2H, CH_2, d, 6.7Hz), 3.86 (1H, 2-CH, t, 7.0Hz), 4.83 (1H, OH, s), 6.85 (2H, ArH, d, 8.6Hz), 7.20 (2H, ArH, d, 8.6Hz)

2.2.2. Enzymatic hydrolysis of 2-cyano-3-(p-hydroxyphenyl)-propionitrile (13)

The enzymatic hydrolysis of **13** was performed using *Rhodococcus erythropolis* NCIB 11450 cells (2.2.2.1.) and the cell-free extract (2.2.2.2.; scheme 8).

At first, the aim of these experiments was to identify their products. In the hydrolysis catalysed by cells the obtained product had the characteristic amide proton signals in ^{1}H-NMR spectrum. The GC/MS experiment confirmed presence of 2-cyano-3-(*p*-hydroxyphenyl)-



Scheme 8. Enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile.

propionamide (**16***), which was also confirmed by TLC after the synthesis of **16**. It was the similar situation as with **1** and **26** as substrates (2.1.2. and 2.3.3. respectively); the cyano-amide was also the product here.

The product of the analogous reaction with the cell-free extract was similar (**16****). It was not surprising since the cell-free extract did not contain any amidase activity, and contained NHase activity.

The yield in the cells reaction was 49%, similarly to the one of the hydrolysis of **1** (51%). However, it might have been much higher. It was very high in further attempts (quantitative conversion after 1 h – TLC), but obtained products were not purified on silica gel columns, due to the risk of their probable racemisation at the α–carbon atom. The hydrogen atom placed there is acidic, since it's attached to the carbon atom bound to the cyano and the amino group as well. So there was a need to take obtained impure **16** directly to further conversions in order to avoid racemisation.

The reaction with the cell-free extract was not completed (TLC) even after 42 hours, and the crude mixture obtained after the reaction was taken to further reactions due to reasons mentioned above. Such an inefficient hydrolysis of **13** was caused by small enzymatic activity of the cell-free extract.

The next tasks were: 1) the synthesis of the racemic product (for seeking an e.e. assay and further chemistry purposes), **16**; 2) the conversion of obtained cyano-amides **16*** and **16**** into tyrosines **20*** and **20****. It might be also an indirect method of determination of e.e.'s of products of these reactions.

2.2.2.1. Enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13; with *Rhodococcus erythropolis* NCIB 11450 cells)

The mixture of 104mg (0.547mmol) of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**) and 1.00g of *Rhodococcus erythropolis* NCIB 11450 cells in 200ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 °C for 2 hours. The mixture was drained through hyflo (then hyflo was washed with 100ml of methanol; these washes were combined with water solution), saturated with $(NH_4)_2SO_4$, and extracted 4 x 100ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, drained and solvents were evaporated. The obtained mixture was purified

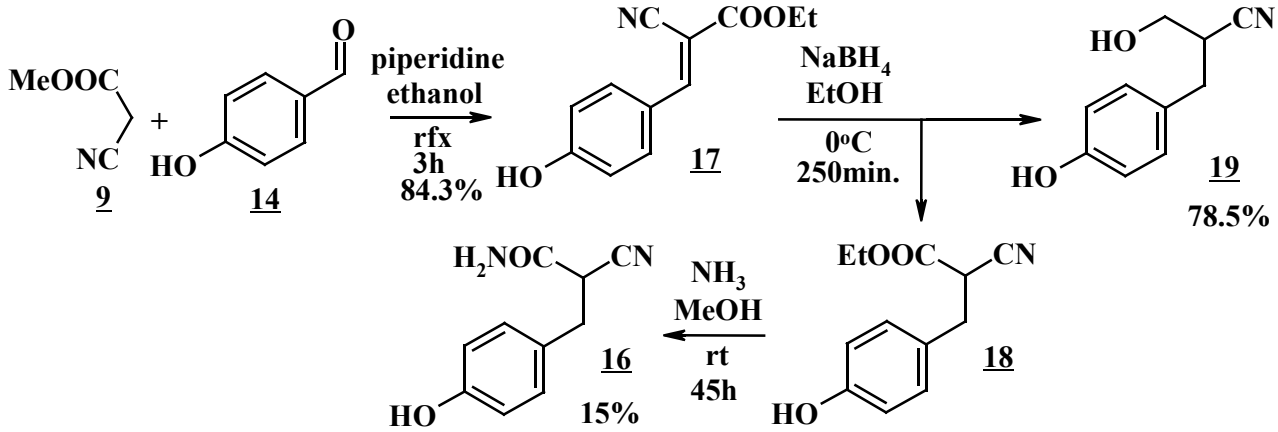
on a silica gel column using ethyl acetate - heptane 1:2 (v/v) as an eluent. 51mg (0.27mmol; 49% yield) of white solid product identified (TLC, ^{1}H-NMR, GC/MS) as 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16***).

2.2.2.2. Enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13; with cell free extract from *Rhodococcus erythropolis* NCIB 11450)

The mixture of 109mg (0.573mmol) of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**) and 9.75ml of the cell free extract from *Rhodococcus erythropolis* NCIB 11450 in 50ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 °C for 42 hours. The mixture was acidified with conc. $HCl_{(aq)}$, saturated with $(NH_4)_2SO_4$, 50ml of water were added, and the mixture was extracted with 100ml and 5 x 50ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, and drained. The solvent was evaporated; 176mg of crude 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16****; TLC) were obtained.

2.2.3. Synthesis of 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (16)

The synthesis of **16** was performed via the Knoevenagel condensation of methyl cyanoacetate (**9**) with *p*-hydroxybenzaldehyde (**14**) leading to ethyl 2-cyano-3-(*p*-hydroxyphenyl)-acrylate (**17**), its subsequent reduction to ethyl (R,S)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**), and the final conversion into (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16**; scheme 9).



Scheme 9. Synthesis of (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide.

The first step of this synthesis was the Knoevenagel condensation of **9** and **14** to **17**.

The first attempt (2.2.3.1.)[14] was performed analogously to the syntheses of **7** (2.1.1.1.), **10** (2.1.3.1.) and **15** (2.2.1.1.). It gave approximately 64% conversion (^{1}H-NMR). Although it is a quite good yield, the method is not a convenient one, since it is very difficult to separate non-reacted **14** and **17**, due to almost same R_f's on silica gel. The conversion was not higher (e.g. almost quantitative, like in the synthesis of **15**) due to low solubility of **9** in toluene. However, it was much better than in the synthesis of **7** (42.5%), since aromatic aldehydes are much more active in this

reaction, than aliphatic aldehydes.

The second attempt (2.2.3.2.)[18] gave very good, 84.3%, yield. The transestrification (exchange of methyl groups from the ester moiety and ethanol from solvent) occurred at the conditions used in this synthesis, but it did not play any important role in the further synthesis. In the similar synthesis of **30** (2.3.2.1.) the transestrification didn't occur, the time of the reaction was shorter there, and the room temperature was applied in that case comparing to 3 times longer reaction time and reflux conditions used in the synthesis of **17**. Only one of the *E,Z* isomers of **17** was formed. This step was performed similarly to the synthesis of **10** (2.1.3.1.; 67.2% yield) and **30** (100% yield). The yields of the condensations with aromatic aldehydes were higher here due to higher reactivity of aromatic aldehydes mentioned above.

The second step of this synthesis was the reduction of **17** to **18**. That was again the limiting step of this synthesis.

The first attempt (2.2.3.3.) was performed using benzaldehyde with 1,2-phenylenediamine similarly to the syntheses of **8** (2.1.1.4.), **11** (2.1.3.2.), and **28** (2.3.1.2). It did not give the quantitative conversion; it was very difficult to determine it. It was not possible to separate **18** from side-products formed by used substrates.

The second attempt (2.2.3.4.) was performed using $NaBH_4$[16], similarly to the syntheses of **8** (2.1.1.3.) and **13** (2.2.1.3.). It led mostly to **19** with 78.5% yield. It meant that $NaBH_4$ had reduced not only the double carbon-carbon bond, but also the ester moiety to primary alcohol of **19**, surprisingly. Desired **18** was also formed, but only with 15% yield. The yield in this synthesis were determined after the conversion of **18** into **16**, which was almost quantitative (TLC).

The last step of this synthesis was the conversion of **18** into **16** (2.2.3.5.) similar to syntheses of **4** (2.1.3.4.), **29** (2.3.2.3.), **37** (3.1.1.4.) and **37*** (3.1.2.3.).

The whole synthesis of **16** gave 12.6% yield. It may be enlarged by applying a different system to the reduction of **17** to **18**.

Obtained **16** was used for seeking a method of determining of e.e in the similar way to **29** (2.3.2.). It was performed on chiral HPLC columns. AD Chiralpack, OB Chiralcel, OD Chiralcel and OJ Chiralcel columns were tested using different mixtures of isopropanol and heptane using UV detection (220 and 280 nm), different flow ratios were tested. However, no separation of both enantiomers was found. There were also attempts done using chiral GC on CP-chirasil-Dex CB (25m x 0.25mm x 0.25m) and Gamma Dex 120TM (30m x 0.25mm x 0.25m) columns, but no separation was observed at different temperatures, either.

2.2.3.1. Synthesis of methyl 2-cyano-3-(*p*-hydroxyphenyl)-acrylate (**17**; in toluene)

841mg (6.89mmol) of *p*-hydroxybenzaldehyde (**14**), 0.700ml (787mg; 7.94mmol) of methyl 2-cyanoacetate (**9**), 22mg of D,L-alanine, 0.45ml of acetic acid and 22ml of toluene were stirred in reflux conditions under a Dean-Stark trap for 50min. 10ml of THF and 10ml of ethyl acetate were added, and the mixture was extracted with 40ml of water. The water layer was extracted with 20ml of ethyl acetate and 2 x 20ml of diethyl ether. All the organic layers were combined and dried over Na_2SO_4. The crude product obtained after draining and evaporation of solvents was purified on a silica gel column using heptane - ethyl acetate 2:1 (v/v) as an eluent. The mixture of desired methyl 2-cyano-3-(*p*-hydroxyphenyl)-acrylate (**17**) with starting *p*-hydroxybenzaldehyde (**14**; with 64% conversion calculated from ^{1}H-NMR spectrum) were obtained.

2.2.3.2. Synthesis of ethyl 2-cyano-3-(*p*-hydroxyphenyl)-acrylate (**17**; in ethanol)

841mg (6.89mmol) of p-hydroxybenzaldehyde (**14**), 0.700ml (787mg; 7.94mmol) of methyl 2-cyanoacetate (**9**), 5 drops of piperidine and 10ml of ethanol were stirred in reflux conditions for 3 hours. Then 40ml of cold water were added, and the obtained mixture was drained. 1.26g (5.81mmol) of yellow crystals of ethyl 2-cyano-3-(p-hydroxyphenyl)-acrylate (**17**) were obtained with 84.3% yield.

^{1}H-NMR (200MHz, CD_3OD): δ 1.32 (3H, CH_3, t, 7.0Hz), 3.84 (1H, OH, s), 4.27 (2H, CH_2, q, 4.0Hz), 6.87 (2H, ArH, d, 8.8Hz), 7.92 (2H, ArH, d, 8.8Hz), 8.16 (1H, CH, s)

2.2.3.3. Synthesis of ethyl (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**; reduction with benzaldehyde)

400mg (1.84mmol) of ethyl 2-cyano-3-(*p*-hydroxyphenyl)-acrylate (**17**), 0.230ml (240mg; 2.27mmol) of benzaldehyde, 232mg (2.15mmol) of 1,2-phenylenediamine and 19ml of ethanol were stirred at room temperature for 168 hours. The solvent was evaporated; and the residue was purified on a silica gel column using heptane - ethyl acetate 2:1 (v/v) as an eluent. 498mg of impure solid ethyl (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**) were obtained.

2.2.3.4. Synthesis of ethyl (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**; reduction with $NaBH_4$)

429mg (1.98mmol) of ethyl (*E*)-2-cyano-3-(*p*-hydroxyphenyl)-propenoate (**17**) were dissolved 7.6ml of ethanol and stirred with cooling in an ice-salt bath. Then 141mg (3.73mmol) of $NaBH_4$ were added in 4 portions during 20min. The mixture was stirred with cooling in the ice-salt

bath for 230min., and quenched with 1.0ml of conc. $HCl_{(aq)}$. Then 10ml of water were added, the mixture was saturated with $(NH_4)_2SO_4$, and extracted 5 x 10ml of ethyl acetate. Combined organic layers were extracted with 12ml of 1M $NaOH_{(aq)}$, and dried over Na_2SO_4. After draining and evaporation of the solvent, 394mg of crude solid mixture of mostly (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-1-propanol (**19**), and also desired ethyl (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**) were obtained.

2.2.3.5. Synthesis of (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide (16)

279mg of crude mixture of ethyl (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionate (**18**) with (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-1-propanol (**19**) were dissolved in 50ml of 7M NH_3 in methanol. Mixture was stirred in a stoppered flask at room temperature for 26 hours, solvent was evaporated, next 30ml of ammonia solution were added, and reaction was continued for next 19 hours as above. Then solvent was evaporated; and obtained mixture was purified on a silica gel column using ethyl acetate - heptane 1:1 (v/v) as an eluent. At first 195mg (1.10mmol; min 78.5% yield calculated from ethyl (*E*)-2-cyano-3-(*p*-hydroxyphenyl)-propenoate, **17**) of (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-1-propanol (**19**) were eluted, then 40mg (0.21mmol; min. 15% yield calculated from ethyl (*E*)-2-cyano-3-(*p*-hydroxyphenyl)-propenoate, **17**) of (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16**) were eluted.

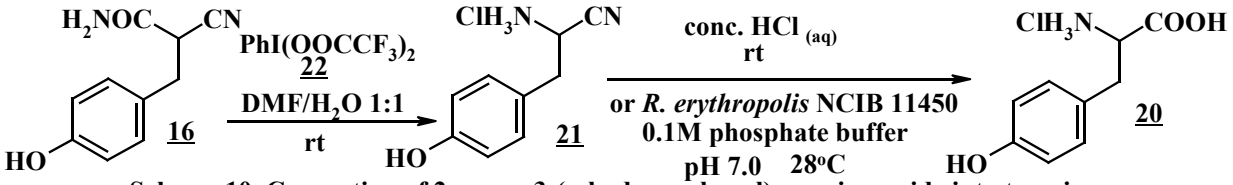
(*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-1-propanol (**19**) ^{1}H-NMR (300MHz, $(CD_3)_2CO$): δ 2.75-2.93 (2H, 3-CH_2, m), 2.93-3.06 (1H, CH, m), 3.64-3.80 (2H, 1-CH_2, m), 4.54 (1H, 1-OH, bs), 6.77 (2H, ArH, d, 8.4Hz), 7.14 (2H, ArH, d, 8.4Hz), 8.33 (1H, ArOH, bs); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$): δ 35.1 (C-3), 38.6 (C-2), 62.9 (C(OH)-1), 116.9 (C-3'Ar), 122.4 (CN), 129.8 (C-1'Ar), 131.7 (C-2'Ar), 157.9 (C(OH)-4'Ar)

(*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16**) ^{1}H-NMR (200MHz, DMSO-d_6): δ 2.87-3.09 (2H, CH_2, m), 3.71-3.82 (1H, CH, dd, 2.0Hz, 6.5Hz), 6.63 (2H, ArH, d, 8.3Hz), 7.01 (2H, ArH, d, 8.3Hz), 7.40 (1H, NH, bs), 7.70 (1H, NH, bs), 9.30 (1H, OH, s)

2.2.4. Conversion of 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (16), obtained during enzymatic hydrolyses, into tyrosine (20)

The conversion of **16** into **20** was led via the Hoffman rearrangement into tyrosine nitrile hydrochloride (**21**), and its hydrolysis into **20** (scheme 10).

The first task here was the synthesis of iodosobenzene bis-(trifluoroacetate) (**22**; 2.2.4.1.). It is a very useful reagent for the Hoffman rearrangement. Classical conditions (Br_2, NaOH) could not be applied here, due to the racemisation of α–cyanoamides. **22** is able to convert them into



Scheme 10. Converting of 2-cyano-3-(*p*-hydroxyphenyl)-propionamide into tyrosine.

α-aminonitrile hydrochlorides in mild conditions (pH 1-3 in water)[19] with retention of configuration. There are some more trivalent-iodine compounds of those properties, e.g. iodosobenzene (PhIO) or hydroxy-(tosyloxy)-iodobenzene (PhI(OTs)OH).

The conversions of amides into amines used to require 5-fold molar excess of **22** (TLC), although the literature procedure[19] required 1 eq. of **22**.

The cyanoamides **16*** and **16**** obtained after enzymatic hydrolyses of **13** (with *Rhodococcus erythropolis* NCIB 11450 cells, 2.2.2.1.; and the cell-free extract, 2.2.2.2.; respectively) were converted into corresponding aminonitrile hydrochlorides **21***, and **21**** using reagent **22**. In the following step, they were hydrolysed to corresponding tyrosine hydrochlorides **20***, and **20**** using concentrated $HCl_{(aq)}$ (2.2.4.2. and 2.2.4.3., correspondingly).

The tyrosine samples were analysed using HPLC and 1-nitroso-2-naphthol assays for determination of total amounts of **20**; and the enzymatic assay for determining amounts of L-isomer (2.2.4.4.).

The described conversions gave very small yields (0.85 and 0.75%, respectively). Limiting steps there were probably the Hoffman rearrangements, although quantitative conversions were reached (TLC). Probably also undesired oxidations with **22** occurred, since aromatic moieties may be sensitive to these conditions. It excludes the synthetic use of trivalent iodine compounds with aromatic compounds.

In both cases (*R*)-isomer is formed in small excess (e.e.s 1.6% and 8.4% after conversions to tyrosines, respectively). It is not surprising, since the same NHase is responsible for this process. Different e.e.s may be due to the presence of other enzymes in the *R.erythropolis* strain. The real e.e.s may be higher, if we consider the potential racemisation of an obtained product and the products of its conversion into the amino acid. The solution of such problem would be finding of a direct method of determining e.e of the formed amido nitrile.

The e.e.s obtained in the analogous reactions starting from **26** (50% and 70%, correspondingly) were much higher. It may mean, that the presence of *p*-hydroxyl group may decrease the (*R*)-stereoselectivity of the enzymatic hydrolysis catalysed by *R. erythropolis*.

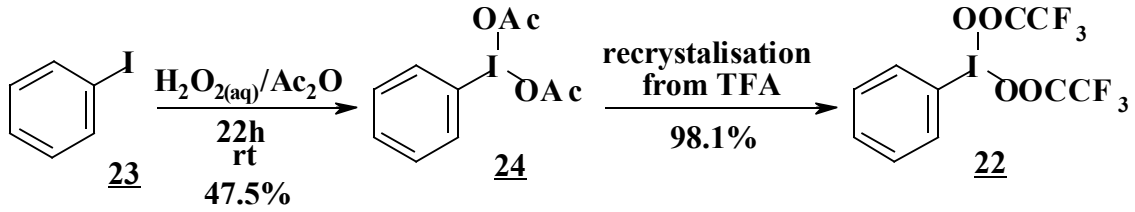
The conversions of racemic **16** into **21** (2.2.4.5.1.) and **29**s into **32**s via corresponding **33**s (2.3.4. and 2.3.4.1.) were performed similarly, and similarly gave very small yields.

The (*R*)-selectivity of the above described reactions and very small yields excludes the use of this synthetic route in the syntheses of labelled amino acids.

It was also worthy to investigate the enzymatic resolution of racemic **21** (2.2.4.5.). It might be an useful tool for enriching of obtained **20** with L-isomer.

2.2.4.1. Synthesis of iodosobenzene bis-(trifluoroacetate) (22)

The synthesis of iodosobenzene bis-(trifluoroacetate) (**22**) was performed via the oxidation of iodobenzene (**23**) to iododsobenzene bisacetate (**24**), and the transacylation into **22** (scheme 11).



Scheme 11. Synthesis of iodosobenzene bis-(trifluoroacetate).

The first step of this synthesis was the oxidation of **23**, leading to **24**[20] in the presence of acetic anhydride (2.2.4.1.1.). It gave 47.5% yield.

The second step was the transacylation of **24** into **22**[21] by simple recrystallisation in TFA (2.2.4.1.2.). It gave excellent 98.1% yield. Obtained **22** had the melting point of 118-121°C, comparing to the literature[22,23] one of 119-126°C.

The whole synthesis of reagent **22** gave 46.6% overall yield.

2.2.4.1.1. Synthesis of iodosobenzene bisacetate (24)

38ml of 35% $H_2O_{2(aq)}$ and 160ml of acetic acid anhydride were stirred and heated in 40 °C under a cooler for 4h. (After 20min. the mixture started to boil, boiling lasted 10min.) Afterwards, 14.0ml (25.5g; 12.5mmol) of iodobenzene (**23**) were added, and the mixture was stirred at room temperature for 22h. Solvents were evaporated, 19.1g (max. 5.93mmol) of white iodosobenzene bisacetate (**24**) were obtained with 47.5% yield.

^{1}H-NMR (200 MHz, D_2O): δ 1.84 (6H, CH_3, s), 7.26-7.36 (2H, *m*-ArH, m), 7.43-7.52 (1H, *p*-ArH, m), 7.83-7.91 (2H, *o*-ArH, m)

2.2.4.1.2. Synthesis of iodosobenzene bis-(trifluoroacetate) (22)

5.00g (15.5mmol) of iodosobenzene bisacetate (**24**) had been stirred with 10.0ml of TFA in a stoppered vial with heating, until whole substrate was dissolved. Then the vial was kept open at room temperature for 5h, TFA was evaporated at room temperature. The obtained mixture was purified by washing 2 x 1ml of diethyl ether. 6.54g (15.2mmol) of white crystals of iodosobenzene bis-(trifluoroacetate) (**22**) were obtained with 98.1% yield. The melting point was 118-121°C.

^{1}H-NMR (200 MHz, D_2O): δ 7.35-7.47 (2H, *m*-ArH, m), 7.51-7.61 (1H, *p*-ArH, m), 7.98-8.05 (2H,

o-ArH, m)

2.2.4.2. Converting of 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (16*) obtained after enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13) with *Rhodococcus erythropolis* NCIB 11450 cells into tyrosine (20*)

131mg (max. 0.445mmol) of crude 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16***; obtained after the enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**; 2.2.2.1.) with *Rhodococcus erythropolis* NCIB 11450 cells) were dissolved in 7.0ml of water. 7.0ml of acetonitrile and 942mg (2.19 mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 43h, and 15ml of water and 2.0ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 20ml of diethyl ether. The combined organic layers were extracted with 22ml of 1.1M $HCl_{(aq)}$. The combined water layers were evaporated. The obtained solid residue was dissolved in 10ml of conc. $HCl_{(aq)}$, and stirred for 45h. The solvent was evaporated and washed 2 x 5ml of acetone. The crude mixture containing 4.2μmol of D-tyrosine of 1.6% e.e. tyrosine (**20***; TLC) were obtained with 0.85% yield starting from corresponding dinitrile **13**.

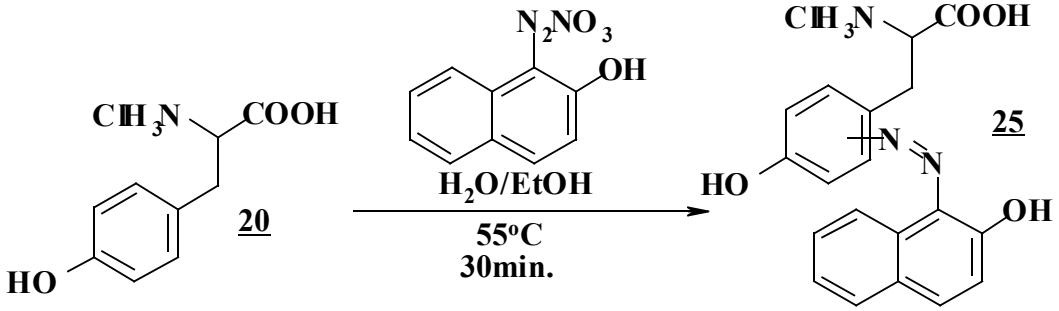
2.2.4.3. Converting of 2-cyano-3-(p-hydroxyphenyl)-propionamide (16**), obtained after enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (13) with cell free extract from *Rhodococcus erythropolis* NCIB 11450, into tyrosine (20**)

141mg (max. 0.458mmol) of crude 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16****; obtained after the enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**; 2.2.2.2.) with the cell free extract from *Rhodococcus erythropolis* NCIB 11450) were dissolved in 5.0ml of water. 5.0ml of acetonitrile and 929mg (2.16mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 28.5h, and 15ml of water and 2.0ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 20ml of diethyl ether. The combined organic layers were extracted with 22ml of 1.1M $HCl_{(aq)}$. The combined water layers were evaporated. The obtained solid residue was dissolved in 10ml of conc. $HCl_{(aq)}$, and stirred for 45h. Then the solution was extracted 3 x 5ml of ethyl acetate. The water layer was evaporated and washed 2 x 5ml of acetone. The crude mixture containing 3.52μmol of D-tyrosine of 8.4% e.e. (**20****; TLC) were obtained with 0.70% yield starting from corresponding dinitrile **13**.

2.2.4.4. Analysis of obtained amino acid samples

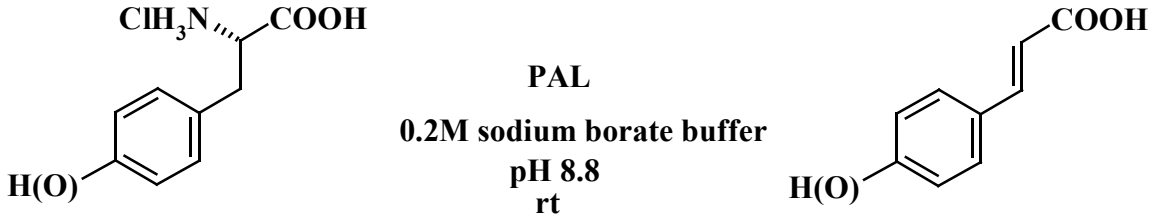
Analyses of each sample were made using HPLC for determination of amounts of D-, and L-isomers of tyrosine (**20**) and phenylalanine (**32**) with UV-detection at 254nm.

Whole amounts of both **20** enantiomers were determined using the 1-nitroso-2-naphthol (forms a diazo compound **25** with tyrosine; it is optical density at wavelength 450nm was measured to determine concentrations of tyrosine) assay (scheme 12; 2.2.4.4.1.)[24].



Scheme 12. 1-nitroso-2-naphthol assay for determination of tyrosine.

The amounts of the L-isomers of the amino acids were determined using L-phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5; scheme 13).



Scheme 13. Enzymatic assay for determination of amount of L-amino acid.

PAL is an enzyme, that converts stereospecifically L-phenylalanine, or L-tyrosine, to (*E*)-cinnamic acid, or to (E)-coumaric acid. A conversion may be masured by a determination of an absorbance increase at the wavelength of 290 nm (2.2.4.4.2.).

2.2.4.4.1. Determination of amount of tyrosine (20) with 1-nitroso-2-naphthol assay

500µl of 0,1% 1-nitroso-2-naphthol solution in ethanol, 500µl of solution of 0,5% $NaNO_2$ in 12% HNO_3, tyrosine (**20**) sample to be assayed, and water up to the total volume of 2.000ml. The mixture was incubated at 55 °C for 30min in a stoppered tube, then cooled to room temperature, and 5.0ml of 1,2-dichloroethane were added. After shaking, the mixture was centrifuged. The absorbance of the water layer was measured at 450nm. The equation of the calibration line:

$$m_{tyr}[\mu g] = 46.1 * A + 2.29$$

2.2.4.4.2. Determination of amounts of L-amino acids using PAL assay

The reaction mixture: sample to be assayed, 20μl of 10-fold diluted PAL from *Rhodotorula glutinis* (of 4.4 U/ml activity; purchased from Sigma) solution, in 0.2M sodium borate buffer, pH 8.8, up to the total volume of 3.000ml. Reaction was carried at room temperature. An increase of the absorbance at 290nm was measured. 1μmole of L-phenylalanine caused an increase of the absorbance equal to 0.548 per hour, the similar value for L-tyrosine was 0.306.

2.2.4.5. Enzymatic resolution of tyrosine nitrile (**21**) with *Rhodococcus erythropolis* NCIB 11450 cells

It was very interesting to investigate the enzymatic hydrolysis of racemic tyrosine nitrile (**21**) with *Rhodococcus erythropolis* NCIB 11450 cells to see, whether an enzymatic hydrolysis may be applied for further enriching of tyrosine (**20**) (obtained from 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16**) produced during an enzymatic hydrolysis of 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**)) with one of the enantiomers.

There is some evidence of enzymatic resolutions of α-amino nitriles[25,26,27,28]; however, there is no corresponding data for **21**. In almost all cases hydrolysis of L-isomer was preferred[27]; which was not surprising, due to possible further incorporation of a formed L-amino acid into bacteria. There is just one known exception of this rule – alanine nitrile hydrolysed by *Rhodococcus rhodochrous* PA-34 cells[27]. In contrary, the hydrolysis of racemic α-aminopropionitrile with *Acinetobacter* sp. gave an excess of L-alanine[28].

The first task here was to synthesise racemic **21** (2.2.4.5.1.). It was performed using the Hoffman rearrangement starting from racemic **16** in the similar manner as with products of enzymatic hydrolyses of **13** (2.2.4.) and **28** (2.3.3.). The obtained product was then hydrolysed to **20*****, using *R. erythropolis* NCIB 11450 cells (2.2.4.5.2.; scheme 10).

The analyses of obtained **20*** were performed as described previously (2.2.4.4.).

The yield of whole synthesis starting from racemic amido nitrile **16** was very small (2.7%) due to the decomposition of the aromatic moiety in the presence of the oxidising agent **22**. This problem also occurred with the analogous synthesis of **32*** (2.3.5.) starting from racemic **29** followed by the microbial hydrolysis of formed phenylalanine nitrile **33** (0.81% yield). The similar synthetic route was also applied to amido nitriles **16** (2.2.4.) and **29** (2.3.3) obtained after the enzymatic hydrolyses of corresponding dinitriles, the hydrolyses of the formed amino nitriles were performed in concentrated HCl. In each case yields were even lower, than in the conversion of racemic **16** into **20***.

Surprisingly, the bacterial resolution of racemic tyrosine nitrile **21** seems to be slightly (15%

e.e.) (*R*)-selective. The similar phenylalanine nitrile **33** resolution gives also *R*-enantiomer of amino acid in excess (69%). It may be common for aromatic amino acids, also the presence of *p*-hydroxyl group may decrease the stereoselectivity of this process.

However, a very small yield and the (*R*)-selectivity also eliminate this synthetic route if we consider the syntheses of isotopically labelled amino acids.

2.2.4.5.1. Synthesis of (*R,S*)-tyrosine nitrile (21)

31mg (0.15mmol) of (*R,S*)-2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**16**) were dissolved in 1.0ml of water. 1.0ml of acetonitrile and 312mg (0.726mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 17h, and 3ml of water and 0.5ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 5ml of diethyl ether. The combined organic layers were extracted with 5.5ml of 1.1M $HCl_{(aq)}$. The combined water layers were evaporated. Crude (*R,S*)-tyrosine nitrile hydrochloride (**21**) were obtained.

2.2.4.5.2. Enzymatic hydrolysis of (*R,S*)-tyrosine nitrile (21) with *Rhodococcus erythropolis* NCIB 11450 cells

The mixture of obtained (2.2.4.5.1.) crude (*R,S*)-tyrosine nitrile (**21**) and 202mg of *Rhodococcus erythropolis* NCIB 11450 cells in 2.00ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 ºC for 79 hours. The reaction was ended by acidifying with 1 drop of conc. $HCl_{(aq)}$, and the mixture was centrifuged; the supernatant was loaded to an Amberlit IR-120 (H^+) cation-exchange column. Salts were washed out with water, and the amino acid was eluted with 0.3M $NH_{3(aq)}$. The solvent was evaporated. The crude mixture containing 0.40μmol of D-tyrosine of 15% e.e. (**20*****; TLC) were obtained with 2.7% yield starting from the corresponding racemic cyano-amide **16**.

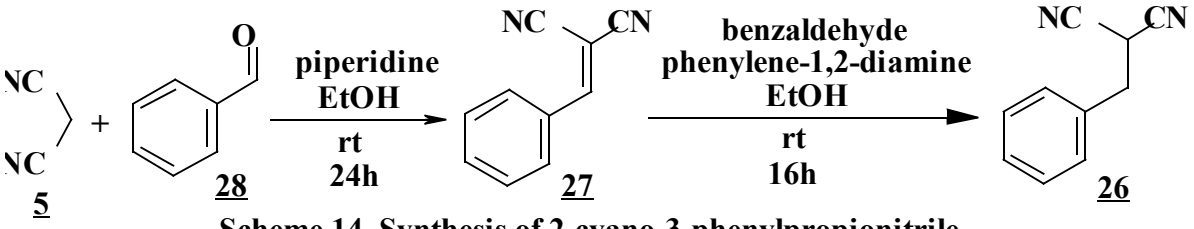
2.3. Investigations of 2-cyano-3-phenylpropionitrile as a substrate

2-cyano-3-phenylpropionitrile (**26**) was another interesting substrate for these investigations. If the cyano-amide was formed during an enzymatic hydrolysis, as in the case of **13** (2.2.2.), it may be then similarly converted into phenylalanine. It may be a novel, interesting way of synthesis of this amino acid useful e.g. for labelling with different isotopes.

2.3.1. Synthesis of 2-cyano-3-phenylpropionitrile (26)

The synthesis of 2-cyano-3-phenylpropionitrile (**26**; scheme 14) was performed via the

Knoevenagel condensation of malonodinitrile (**5**) with benzaldehyde (**28**), followed by the reduction of formed 2-cyano-3-phenylpropionitrile (**27**). This synthesis was performed in our group by Christien Schortinghuis.



Scheme 14. Synthesis of 2-cyano-3-phenylpropionitrile.

The first step of this synthesis was the Knoevenagel condensation of **5** and **28**[14] to give **27** with quantitative yield. (2.3.1.1.). The synthesis of **17** (2.2.3.1.) was performed in the similar manner, but the yield there (64%) was significantly smaller. It seems, that phenyl derivative **27** is much more easily formed, that the *p*-hydroxyphenyl one **17**.

The subsequent step was the reduction of **27** to **26** using benzaldehyde/ 1,2-phenylenediamine similarly to the reductions of **7** to **8** (96.0%; 2.1.1.4.), **10** to **11** (24.3% yield, 2.1.3.2.), and **17** to **18** (2.2.3.3.). It gave very good 90% yield.

The yield of the whole synthesis of **26** was very high – 90%.

2.3.1.1. Synthesis of 2-cyano-3-phenylacrylonitrile (27)

Malonodinitrile (**5**, 5.18g, 78.5mmol), benzaldehyde (**28**, 9.66g, 91.1mmol) and piperidine (0.66g) in ethanol (150ml) were stirred for 24h at room temperature. 250 ml of water were added to the reaction mixture, which was then extracted with ethyl acetate (3 x 50ml). The organic layer was dried over $MgSO_4$, drained, and evaporated. 12.9g (78.5mmol) of white solid 2-cyano-3-phenylacrylonitrile (**27**) were obtained with 100% yield.

^{1}H NMR (100MHz, $CDCl_3$): δ 7.26-7.65 (3H, *m*- and *p*-ArH, m), 7.78 (1H, CH, s), 7.87-7.97 (2H, *o*-ArH, m)

2.3.1.1. Synthesis of 2-cyano-3-phenylpropionitrile (26)

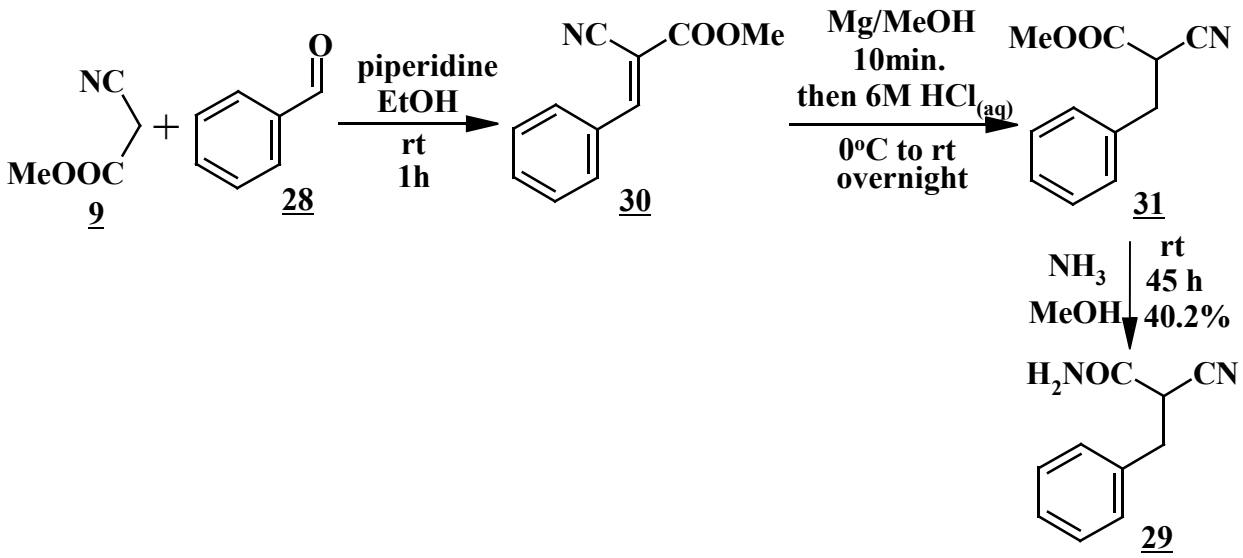
2-cyano-3-phenylacrylonitrile (**27**, 1.08g, 6.35mmol), phenylene-1,2-diamine (0.72g, 6.9mmol) and benzaldehyde (0.71g, 6.7mmol) in ethanol (65ml) were stirred at room temperature for 16h. Ethanol was evaporated, 50ml of dichloromethane were added to the remaining yellow product. The solvent was evaporated after filtration. 0.89g (5.7mmol) of pale solid 2-cyano-3-phenylpropionitrile (**27**) with 90% yield.

^{1}H NMR (100MHz, $CDCl_3$): δ 3.23 (2H, CH_2, d, 6.9Hz), 3.84 (1H, CH, t, 13.9Hz), 7.26-7.41 (5H, ArH, m)

2.3.2. Synthesis of (R,S)-2-cyano-3-phenylpropionamide (29)

The experiments of Christien Schortinghuis in our group showed, that the product of the bacterial hydrolysis of 2-cyano-3-phenylpropionitrile (**26**) using *R. erythropolis* NCIB 11450 cells and the cell-free extract is also the amido-nitrile: 2-cyano-3-phenylpropionamide (**29**). The synthesis of the racemic latter was necessary for looking for a good e.e. assay and further chemistry purposes.

The synthesis of (*R,S*)-2-cyano-3-phenylpropionamide (**26**; scheme 15) was performed from methyl (*R,S*)-2-cyano-3-phenylpropionate (**31**, obtained previously in our group by Christien Schortinghuis by the Knoevenagel condensation of methyl 2-cyanoacetate (**9**) with benzaldehyde (**28**), and subsequent reduction of obtained methyl 2-cyano-3-phenylacrylate (**30**)) using ammonia.



Scheme 15. Synthesis of (*R,S*)-2-cyano-3-phenylpropionamide.

The starting step was the Knoevenagel condensation of **9** and **28** to give **30**

This procedure was analogous to the one used for the synthesis of **10** (67.2% yield; 2.1.3.1.) and **17** (84.3%; 2.2.3.2.), and it gave higher yield – 90.0%. Aromatic aldehydes seem to be more reactive in this process. The transestrification of the methyl group with the ethyl group from the solvent did not occur here, as it was in the synthesis of **17**. The reaction time here was 3 times shorter, and the room temperature was applied instead reflux conditions.

Subsequently, **30** was reduced using Mg/HCl to **31**. The reduction was performed similarly to the reductions of the dinitriles **7** (12.5% yield; 2.1.1.2.), and **15** (5.45%; 2.2.1.2.); however, it gave excellent 93.0% yield.

The last step of this synthesis was the nucleophilic substitution of methoxy group of **31** with the amino group of ammonia, which gave **29**. The procedure (2.3.2.3.) was similar to the ones used for syntheses of **4** (2.1.3.4.), **16** (2.2.3.5.), **37** (3.1.1.4.) and **37*** (3.1.2.3.). However, the yield (40.2%) was lower, than the analogous one obtained in the synthesis of **4** (76.9%).

The overall yield of the synthesis of **29** was 33.6%, which is not excellent, but satisfactory. The last step should be optimised here.

Obtained **29** was used for looking for a method of determining e.e similarly to **16** (2.2.3.). It was performed using chiral HPLC columns. AD Chiralpack, OB Chiralcel, OD Chiralcel and OJ Chiralcel columns were tested using isopropanol/heptane solvents using UV detection at 220 and 254nm, and different flow ratios; but no separation of both enantiomers was found. There were also trials attempted using chiral GC on CP-chirasil-Dex CB (25m x 0.25mm x 0.25m) and Gamma Dex 120TM (30m x 0.25mm x 0.25m) columns, and no separation was observed at different temperatures, too.

2.3.2.1. Synthesis of methyl 2-cyano-3-phenylacrylate (30)

Methyl 2-cyanoacetate (**9**, 5.05g, 51.0mmol), benzaldehyde (**28**, 6.46g, 60.9mmol) and piperidine (0.44g) in ethanol (70ml) were stirred for 1h at room temperature. 200ml of water were added to the reaction mixture; the latter was extracted 3 x 50ml of ethyl acetate. The organic layer was dried over $MgSO_4$, drained, and evaporated. The residue was purified on a silica gel column using heptane-ethyl acetate 4:1 as an eluent. 8.58g (45.9mmol) of methyl 2-cyano-3-phenylacrylate (**30**) were obtained with 90.0% yield.

^{1}H NMR (100MHz, $CDCl_3$): δ 3.95 (3H, OMe, s), 7.49-7.60 (3H, *m*- and *p*-ArH, m), 7.96-8.05 (2H, *o*-ArH, m), 8.27 (1H, CH, s)

2.3.2.2. Synthesis of methyl (*R,S*)-2-cyano-3-phenylpropionate (31)

Methyl 2-cyano-3-phenylacrylate (**30**, 0.994g, 5.31mmol) and Mg (5.15g, 214mmol) in methanol (53ml) were stirred at room temperature for 10min. The reaction mixture was cooled down to 0ºC. Then 6M $HCl_{(aq)}$ solution (98ml) was added to the reaction mixture dropwise with temperature maintained around 0ºC. Afterwards, the reaction mixture was stirred at room temperature overnight, and extracted with 3 x 50ml of diethyl ether. The combined organic layers were washed with brine (40ml), dried over $MgSO_4$, drained, and evaporated. 933mg (4.94mmol) of methyl (*R,S*)-2-cyano-3-phenylacrylate (**31**) were obtained with 93.0% yield.

^{1}H NMR (300MHz): δ 3.19-3.27 (2H, CH_2, m), 3.71-3.78 (1H, CH, m), 3.79 (3H, OMe, s), 7.26-7.35 (5H, ArH, m)

2.3.2.3. Synthesis of (*R,S*)-2-cyano-3-phenylpropionamide (29)

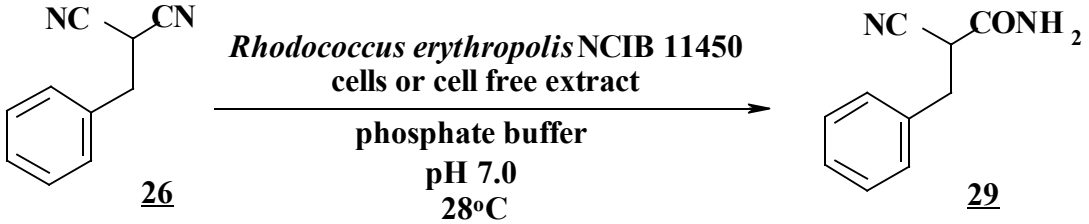
3.96g (21.0mmol) of methyl (*R,S*)-2-cyano-3-phenylpropionate (**31**) were dissolved in 200ml of 7M NH_3 in methanol. The mixture was stirred at room temperature in a stoppered flask for 64h. Then the solvent was evaporated, and the residue was purified on a silica gel column using ethyl acetate - heptane 1:1 (v/v) as an eluent. 1.47g (8.45mmol) of white solid (*R,S*)-2-cyano-

3-phenylpropionamide (**29**) were obtained with 40.2% yield.

^{1}H-NMR (200MHz, DMSO-d_6): δ 2.90-3.13 (2H, CH_2, m), 3.82-3.93 (1H, CH, m), 7.13-7.32 (5H, ArH, m), 7.44 (1H, NH, bs), 7.73 (1H, NH, bs)

2.3.2. Enzymatic hydrolyses of 2-cyano-3-phenylpropionitrile (26)

The enzymatic hydrolyses of **26** were performed using *Rhodococcus erythropolis* NCIB 11450 cells (2.3.2.1.) and the cell-free extract (2.3.2.2.; scheme 15) as well.



26 **29**

Scheme 16. Enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile.

In both cases the obtained compound was 2-cyano-3-phenylpropionamide (**29*** and **29****, respectively; TLC), which had been already proven in our group. It's an analogous situation to the hydrolyses of **1** (with cells; 2.1.2.) and **13** (both with cells and the cell-free extract; 2.2.2.), where cyano-amides **4** and **16** were isolated as the products.

The yield in the cells reaction was almost quantitative after 1.5h – TLC, but the obtained product was not purified on a silica gel column, due to its probable racemisation at the α–carbon atom, as mentioned in the case of the hydrolyses of **13**. Impure **29**‘s were directly taken to further conversions in order to avoid racemisation.

The reaction with the cell-free extract was not completed (TLC) even after 21 hours, and the crude mixture obtained after the reaction was taken to further reactions due to mentioned reasons. It was caused by small enzymatic activity of the cell-free extract.

The next tasks were the conversions of obtained cyano-amides **29*** and **29**** into phenylalanines **32*** and **32****, respectively. It may be indirectly used for determination of e.e.s of the products of these reactions.

2.3.3.1. Enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (26) with *Rhodococcus erythropolis* NCIB 11450 cells

The mixture of 111mg (0.712mmol) of 2-cyano-3-phenylpropionitrile (**26**) and 222mg of *Rhodococcus erythropolis* NCIB 11450 cells in 200ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 °C for 160min. The mixture was acidified with 10ml of conc. $HCl_{(aq)}$, saturated with $(NH_4)_2SO_4$, and extracted 4 x 100ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, drained, and solvents were evaporated. 129mg of white solid, crude

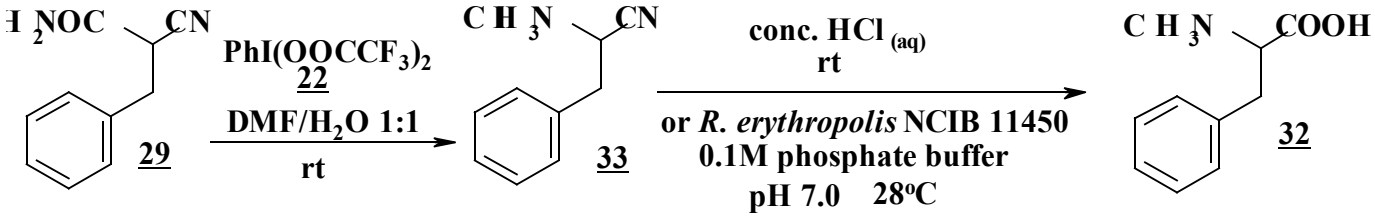
2-cyano-3-phenylpropionamide (**29***; TLC) were obtained.

2.3.3.2. Enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (26) with cell-free extract from *Rhodococcus erythropolis* NCIB 11450

The mixture of 114mg (0.731mmol) of 2-cyano-3-phenylpropionitrile (**26**) and 3.00ml of cell-free extract from *Rhodococcus erythropolis* NCIB 11450 in 50ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 °C for 21 hours. The mixture was acidified with conc. $HCl_{(aq)}$, saturated with $(NH_4)_2SO_4$, 150ml of water were added, and the mixture was extracted with 50ml, 100ml and 3 x 50ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, and drained. The solvent was evaporated, the residue was washed with heptane. 111mg (max 0.638mmol) of crude 2-cyano-3-phenylpropionamide (**29****; TLC) were obtained with max. 87.3% yield.

2.3.4. Conversion of 2-cyano-3-phenylpropionamides (29) obtained in enzymatic hydrolyses of 2-cyano-3-phenylpropionitrile (26) into phenylalanines (32)

The conversions of 2-cyano-3-phenylpropionamides (**29*** and **29****) obtained in the enzymatic hydrolyses of 2-cyano-3-phenylpropionitrile with *Rhodococcus erythropolis* NCIB 11450 cells (2.3.3.1.) and the cell-free extract (2.3.3.2.), respectively; into corresponding phenylalanines **32*** and **32**** (scheme 17) were done via the Hoffman rearrangements of **29*** and **29**** to phenylalanine nitrile hydrochlorides **33*** and **33****, and their subsequent hydrolyses to **32*** and **32****, respectively.



Scheme 17. Converting of 2-cyano-3-phenylpropionamide into phenylalanine.

The cyano-amides **29*** and **29**** obtained after hydrolyses of **26** were converted into corresponding aminonitrile hydrochlorides **33*** and **33**** using the reagent **22**. In following steps they were hydrolysed to corresponding phenylalanine hydrochlorides **32*** and **32**** using concentrated $HCl_{(aq)}$ (2.3.4.1. and 2.3.4.2.). Analyses of obtained samples were done in the similar manner (2.2.4.4.).

These conversions gave very small yields (0.68 and 0.59%, respectively) due to inefficient Hoffman rearrangements.

In both cases (*R*)-isomer was also formed in excess (e.e.s 50% and 70% after conversions to phenylalanines, respectively). It's not surprising, since the same NHase catalyses this reaction.

Different e.e.s may be due to presence of other nitrile-converting enzymes in the *R.erythropolis* strain. The real e.e.s may be higher, due to potential racemisation of the obtained product and products of its conversion into amino acid, which may occur during the synthesis.

The e.e.s obtained in the analogous reactions starting from **13** (1.6% and 8.4%, correspondingly) were significantly lower. It may mean that the presence of *p*-hydroxyl group may decrease the stereoselectivity of the enzymatic hydrolysis catalysed by *R. erythropolis*.

The conversions described in this chapter were performed analogously to conversions of **16**s into **20**s (2.2.4.2. and 2.2.4.3.). The similar Hoffman rearrangement procedures were applied to racemic **16** (2.2.4.5.1.) and **33** (2.3.5.1.). In each case they gave very small yields.

The (*R*)-selectivity of the enzymatic hydrolyses and very small yields excludes the use of this synthetic route in the syntheses of labelled phenylalanines.

It was also worthy to study the enzymatic resolution of racemic **33** (2.3.5.). It may be an useful tool for enriching of obtained **32** with one of enantiomers.

2.3.4.1. Converting of 2-cyano-3-phenylpropionamide (29*) obtained after enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (26) with *Rhodococcus erythropolis* NCIB 11450 cells into phenylalanine (32*)

116mg (max. 0.641mmol) of crude 2-cyano-3-phenylpropionamide (**29***; obtained after the enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (**26**) with *Rhodococcus erythropolis* NCIB 11450 cells) were dissolved in 5.0ml of water. 5.0ml of acetonitrile and 1.04g (2.42mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 46h, and 15ml of water and 2.0ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 25ml of diethyl ether. The combined organic layers were extracted with 27ml of 0.9M $HCl_{(aq)}$. The combined water layers were evaporated. The obtained solid residue was dissolved in 10ml of conc. $HCl_{(aq)}$, and stirred for 128h. The solvent was evaporated, the residue was dissolved in 5ml of 0.3M $NH_{3(aq)}$. The solution was extracted 3 x 5ml of ethyl acetate. The water layer was evaporated and washed with 5ml of acetone. The crude mixture containing 4.4μmol of D-phenylalanine of 50% e.e. (**32***; TLC) were obtained with 0.68% yield starting from corresponding dinitrile **26**.

2.3.4.2. Converting of 2-cyano-3-phenylpropionamide (28**) obtained after enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (26) with cell free extract from *Rhodococcus erythropolis* NCIB 11450 into phenylalanine (32**)

86mg (max. 0.49mmol) of crude 2-cyano-3-phenylpropionamide (**29****; obtained after the enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (**26**) with the cell-free extract from

Rhodococcus erythropolis NCIB 11450) were dissolved in 4.5ml of water. 4.5ml of acetonitrile and 854mg (1.99mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 21h, and 15ml of water and 2.0ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 20ml of diethyl ether. The combined organic layers were extracted with 27ml of 0.9M $HCl_{(aq)}$. The combined water layers were evaporated. The obtained solid residue was dissolved in 10ml of conc. $HCl_{(aq)}$, and stirred for 68.5h. Then the solvent was evaporated, residue was dissolved in 5ml of 0.3M $NH_{3(aq)}$. The solution was extracted 3 x 5ml of ethyl acetate. The water layer was evaporated and washed with 5ml of acetone. The crude mixture containing 3.3μmol of D-phenylalanine of 70% e.e. (**32****; TLC) were obtained with 0.59% yield starting from corresponding dinitrile **26**.

2.3.5. Enzymatic resolution of phenylalanine nitrile (33) with *Rhodococcus erythropolis* NCIB 11450 cells

It was also quite interesting to investigate enzymatic hydrolysis of racemic phenylalanine nitrile (**33**) with *Rhodococcus erythropolis* NCIB 11450 cells in order to know, if enzymatic hydrolysis may be useful for further enriching of phenylalanine (**32**) (obtained from 2-cyano-3-phenylpropionamide (**28**) formed during enzymatic hydrolysis of 2-cyano-3-phenylpropionitrile (**26**)) with one of enantiomers.

It was performed similarly to the enzymatic resolution of tyrosine nitrile (**21**; 2.2.4.5.).

This substrate had already been a substrate for enzymatic resolution[25,26]; however, there is no data of the enantioselectivity of this reaction.

The first step here was the synthesis of racemic **33** (2.3.5.1.). It was done via the Hoffman rearrangement starting from racemic **28**. This procedure was also used for the conversions of **16**s (2.2.4.2. and 2.2.4.3.) and **26**s (2.3.4.) obtained in the enzymatic hydrolyses, into corresponding nitriles. The obtained product was hydrolysed to **33*****, using *R. erythropolis* NCIB 11450 cells (2.3.5.2.; scheme 17).

The analysis of obtained **32***** sample was performed as described previously (2.2.4.5.).

The yield of the whole synthesis starting from racemic amido-nitrile **28** was very small (0.81%) due to the oxidation of the aromatic system in the presence of the agent **22**. This problem also occurred for the similar synthesis of **20***** (2.3.4.) starting from racemic **16** followed by the bacterial hydrolysis of formed tyrosine nitrile **21** (2.7% yield). The analogous synthetic pathway was applied to the amido-nitriles **16** (2.2.4.) and **28** (2.3.4.) obtained after enzymatic hydrolyses of corresponding dinitriles, the hydrolyses of formed amino nitriles were performed in concentrated HCl. In each case yields were also very low.

Surprisingly, the bacterial resolution of racemic phenylalanine nitrile **33** seems to be

(*R*)-selective (69% e.e.). The similar tyrosine nitrile **21** resolution gives also *R*-enantiomer of the amino acid in excess (15%). It may be a rule for aromatic amino acids; the presence of *p*-hydroxyl group may decrease the stereoselectivity of this process.

However, a very small yield and the (*R*)-selectivity also cross out this pathway if we consider the syntheses of the isotopically labelled amino acids.

2.3.5.1. Synthesis of (*R,S*)-phenylalanine nitrile (33)

52mg (0.30mmol) of (*R,S*)-2-cyano-3-phenylpropionamide (**29**) were dissolved in 2.5ml of water. 2.5ml of acetonitrile and 520mg (1.21mmol) of iodosobenzene bis-(trifluoroacetate) (**22**) were added. The mixture was stirred at room temperature for 21h, and 15ml of water and 1.0ml of conc. $HCl_{(aq)}$ were added. The solution was extracted 3 x 25ml of diethyl ether. The water layer was evaporated. Crude (R,S)-phenylalanine nitrile hydrochloride (**33**) was obtained.

2.3.4.2. Enzymatic hydrolysis of (*R,S*)-phenylalanine nitrile (33) with *Rhodococcus erythropolis* NCIB 11450 cells

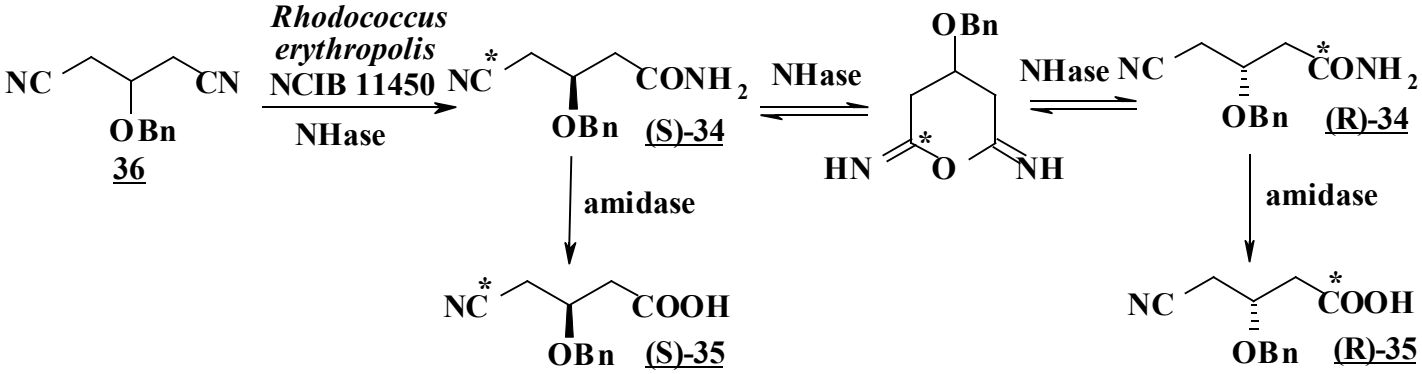
The mixture of obtained (2.3.5.1.) of (*R,S*)-phenylalanine nitrile (**33**) and 99mg of *Rhodococcus erythropolis* NCIB 11450 cells in 2.00ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 ^{o}C for 21 hours. The mixture was centrifuged, solution was acidified with conc. $HCl_{(aq)}$, and loaded on a Dowex 50X2 - an cation exchange resin. Salts were eluted with water, and then phenylalanine (**32*****) was eluted with 0.3M $NH_{3(aq)}$. The solvent was evaporated from the phenylalanine (**32*****) solution, and the residue was dissolved in 10ml of 0.3M $NH_{3(aq)}$. The obtained solution was extracted 3 x 10ml of ethyl acetate. The water layer was evaporated, the residue was washed with 5ml of acetone. The crude mixture containing 2.7μmol of D-phenylalanine of 69% e.e. (**32*****; TLC) were obtained with 0.81% yield starting from the corresponding racemic cyano-amide **28**.

3. Investigations of mechanism of racemisation of 5-cyano-3-hydroxybutyramide in the presence of nitrile hydratase from *Rhodococcus erythropolis* NCIB 11450

Differently 3-substituted glutarodinitriles are the most common compounds investigated in asymmetric hydrolyses of dinitriles[29, 30, 31, 32]. They are very useful compounds for further syntheses, due to the possibility of the formation of 6-membered ring. Due to this fact hydrolyses of such compounds are studied as a potential source of enantiomerically pure building blocks.

In our group racemisation of 3-benzyloxy-5-cyanobutyramide (**34**) in presence of nitrile hydratase from *Rhodococcus erythropolis* NCIB 11450 was observed. It was simply seen by the decreasing of e.e. of the product (3-benzyloxy-4-cyanobutytric acid; **35**) with extending of the time of the enzymatic hydrolysis of 3-benzyloxyglutarodinitrile (**36**) with whole cells.

The probable mechanism of this process via the six-membered ring by-product was proposed (scheme 18).



Scheme 18. Proposed mechanism of decreasing of e.e. of product of hydrolysis of 3-benzyloxyglutarodinitrile.

The aim of this part of work was to verify the proposed mechanism using isotopic methods. If the product labelled specifically with ^{13}C in cyano group was used, the exchange of the label between carbonyl and cyano groups after the treatment with NHase should be observed in ^{13}C-NMR spectra if the proposed mechanism was true. It will be performed using (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37**; non-benzylated compound), which should act in the similar manner.

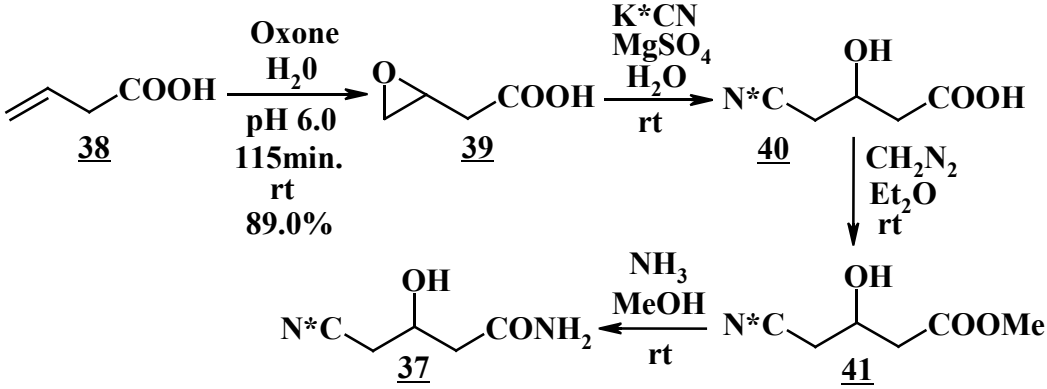
The tasks were: 1) to synthesise (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37**); and 2) to investigate isotopic exchange between carbonyl and cyano groups in **37** using the cell-free extract from *R. erythropolis* NCIB 11450, and whole cells.

3.1. Synthesis of (R,S)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37)

Syntheses of labelled compounds need to be performed on natural isotopic composition at

first. If it gives good results, the synthetic methods may be applied to the syntheses of the labelled compounds.

The synthesis of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37**) was performed via the oxidation of vinylacetic acid (**38**) to (*R,S*)-3,4-epoxybutyric acid (**39**), subsequent nuclephillic substitution of oxygen with [^{13}C]-cyanide leading to (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40**), its methylation to methyl (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyroate (**41**), and the final conversion into (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37**; scheme 19).



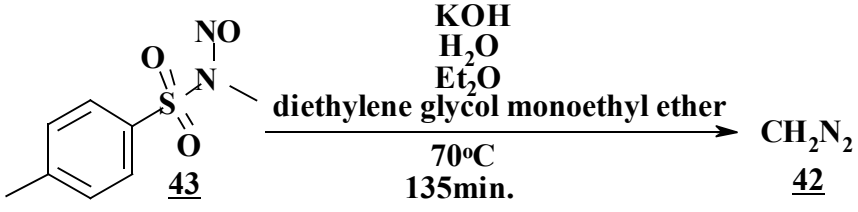
Scheme 19. Synthesis of (*R,S*)-3-hydroxy-4-[5-*C]-cyanobutyramide.

3.1.1. Synthesis of (*R,S*)-3-hydroxy-4-cyanobutyramide (37)

This synthesis was started with the epoxydation of **38** to **39**[33]. Oxone (containing potassium hydrogen persulfate, $KHSO_5$, as an active compound) was used as the epoxydating agent, the reaction was carried at pH 6.0 (3.1.1.1.). If it had been lower, the reaction wouldn't have occurred with satisfactory yield. If it had been higher, Oxone would have been destroyed with the liberation of free oxygen. This procedure gave very good 89.0% yield.

The second step was the conversion of **39** into **40**. It was performed by the substitution of the epoxy oxygen atom with cyanide (at γ–carbon atom), which caused the opening of the 3-membered ring, and the formation of **40**. It was catalysed by $MgSO_4$ as a Lewis acid. This procedure gave 44.1% yield. Syntheses of **40*** (3.1.2.1.) and **40**** (3.1.2.2.) were performed analogously.

The subsequent step was the methylation of **40** to **41** using diazomethane (**42**), which was obtained by a standard procedure (3.1.1.3.; scheme 20) of basic decomposition of Diazald (**43**)[34]. The similar methylation procedure was used for synthesis of **41*** (3.1.2.3.).



Scheme 20. Preparation of diazomethane.

The obtained crude **41** was then converted to **37** (3.1.1.4.) using ammonia analogously to the conversion of **12** into **4** (2.1.2.3.4.), **18** into **16** (2.2.3.5.), **31** to **29** (2.3.2.3.) and **41*** to **37*** (3.1.2.3.).

The last 2 steps gave good, 68.3% yield.

The whole synthesis of **37** gave 30.1% yield calculated from **39**. It was satisfactory enough to be applied to the synthesis of labelled **37***. The most limiting step here was the synthesis of **40**.

The similar synthesis of labelled **37*** (3.1.2.) gave only 6.89% yield surprisingly.

3.1.1.1. Synthesis of (*R,S*)-3,4-epoxybutyric acid (39)

4.89g (15.9mmol) of Oxone were dissolved in 20ml of water, and the pH of the solution was adjusted to 6.0 with 10ml of 1M $KOH_{(aq)}$. Then the solution of 0.750ml (760mg, 8.83mmol) of vinylacetic acid (**38**) in 8.5ml of water was added, and the mixture was stirred at room temperature with maintaining of pH at 6.0 using 1M $KOH_{(aq)}$ (about 21ml) for 115min. Then the solution was acidified to pH 3.0 with conc. $HCl_{(aq)}$, and extracted 5 x 40ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, drained, and the solvent was evaporated. 802mg (max. 7.86mmol) of the colourless oil of (*R,S*)-3,4-epoxybutyric acid (**39**) were obtained with 89.0% yield. ^{1}H-NMR (200MHz, $CDCl_3$): δ 2.53-2.67 (3H, CH_2 + one of the 4-CH_2s, m), 2.82-2.92 (1H, 4- CH_2, m), 3.22-3.35 (1H, CH, m), 8.85 (1H, COOH, bs)

3.1.1.2. Synthesis of (*R,S*)-3-hydroxy-4-cyanobutyric acid (40)

342mg (3.25mmol) of (*R,S*)-3,4-epoxybutyric acid (**39**) were dissolved in 1.65ml of water. 779mg (6.49mmol) of $MgSO_4$ and 160mg (3.27mmol) of sodium cyanide were added. The mixture was stirred at room temperature for 15h. Then the reaction was stopped by acidifying with conc. $HCl_{(aq)}$ to pH 0.9. The mixture was saturated with $(NH_4)_2SO_4$, and extracted 8 x 5.0ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, drained, and the solvent was evaporated. 185mg (1.43mmol) of liquid (*R,S*)-3-hydroxy-4-cyanobutyric acid (**40**) were obtained with 44.1% yield.

3.1.1.3. Preparation of diazomethane (42)

The mixture of 6.00g (107mmol) of KOH, 10ml of water, 10ml of diethyl ether and 35ml of diethylene glycol monoethyl ether was heated at 73 °C. Then the solution of 21.8g (97.3mmol) of Diazal (**43**) in 130ml of diethyl ether was added dropwise during 135min with distilling of diazomethane and diethyl ether into the mixture of 6.0g of KOH and 25ml of diethyl ether. Then more 20ml of diethyl ether were dropped and distilled. The obtained solution of diazomethane (**42**)

in diethyl ether was stored at -20 °C.

3.1.1.4. Synthesis of (*R,S*)-3-hydroxy-4-cyanobutyramide (37)

185mg (1.43mmol) of (*R,S*)-3-hydroxy-4-cyanobutyric acid (**40**) were stirred in 18ml of the diazomethane (**39**)/diethyl ether solution at room temperature without stoppering, until the solvent had evaporated. Then next 15ml of the diazomethane (**42**) solution were added, and the reaction was continued as above. 16.5ml of 7M NH_3 in methanol were added to obtained crude methyl (*R,S*)-3-hydroxy-4-cyanobutyrate (**41**), and the mixture was stirred in a stoppered vial at room temperature for 92h. The solvent was evaporated at room temperature, and next 15.5ml of the ammonia solution were added, and the reaction was continued for more 20h in the similar manner. The residue obtained after evaporation of the solvent was loaded to a silica gel column, and eluted with acetone. 125mg (0.977mmol) of white solid (*R,S*)-3-hydroxy-4-cyanobutyramide (**37**) with 68.3% yield.

^{1}H-NMR (200MHz, $(CD_3)_2CO$): δ 2.38-2.47 (2H, 2-CH_2, m), 2.48-2.73 (2H, 4-CH_2, m), 4.11-4.26 (1H, CH, m), 6.44 (1H, NH, bs), 7.02 (1H, NH, bs); ^{13}C-NMR (75 MHz, CD_3OD): δ 26.3 (C-4), 42.9 (C-2), 65.7 (C-3), 119.1 (CN), 175.6 ($CONH_2$)

3.1.2. (*R,S*)-[5-^{13}C]-3-hydroxy-4-cyanobutyramide (37*)

The synthesis of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***) was performed in the similar manner to the synthesis of (*R,S*)-3-hydroxy-4-cyanobutyramide (**37**; 3.1.1.; scheme 19).

The first step here was the synthesis of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40***; 3.1.2.1.; **40****; 3.1.2.2.). It was performed similarly to the synthesis of (*R,S*)-3-hydroxy-4-cyanobutyric acid (**40**; 3.1.1.2.). Labelled cyanide was added at first in order to obtain better incorporation of expensive ^{13}C isotope, and then after some time non-labelled cyanide was added to convert the rest of non-reacted **39**. However; it didn't increase the incorporation of labelled carbon into product comparing to the incorporation calculated from substrates (27.3% in both cases). The reason was probably equilibrium state here; therefore addition of the labelled substrate some time before addition of the non-labelled one couldn't change the isotopic ratio significantly.

The reaction was performed 2 times, product of the second one (3.1.2.2.) will be used for the synthesis of (*R,S*)-3-benzyloxy-4-[5-^{13}C]-cyanobutyramide (**34***) necessary for further investigations using the same system, where studied phenomenon was discovered in.

The first reaction (3.1.2.1.) did not occur smooth. It probably gave very small yield comparing to the analogous synthesis of **40** (44.1%), and it is responsible for very small yield of the whole synthesis (2.23%), because further steps were quite efficient (68.3% with non-labelled compounds; 3.1.1.4.). The reaction mixture became very dark (normally colour here was dark

yellow) and dense, which did not look promising. Therefore the obtained product was very crude.

The next 2 steps were subsequent conversions to **41*** and **37*** (3.1.2.3.) similar to those used in the synthesis of non-labeled compounds (3.1.1.4.).

The whole synthesis of **37*** gave very small, 2.23% chemical (comparing to 30.1% with non-labelled compound) and 2.28% isotopic yield. Obtained **37*** was 24.8-fold enriched with ^{13}C isotope in the carbon atom of cyano group. Although the small yield of this synthesis, the amount of obtained **37*** was big enough to perform investigations of mechanism of its racemisation catalysed by NHase from *Rhododcoccus erythropolis* NCIB 11450.

3.1.2.1. Synthesis of (*R,S*)-[5-^{13}C]-3-hydroxy-4-cyanobutyric acid (40*; 1st trial)

802mg (7.86mmol) of (*R,S*)-3,4-epoxybutyric acid (**39**), 1.83g (15.2mmol) of $MgSO_4$ and 128mg (1.94mmol) of $K^{13}CN$ (97% of ^{13}C atoms), were stirred at room temperature for 42h, then 338mg (6.90mmol) of NaCN were added, and the mixture was stirred for more 52h, and acidified with conc. $HCl_{(aq)}$ to pH 1.7, saturated with $(NH_4)_2SO_4$, and extracted 7 x 5.0ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, and drained. The solvent was evaporated. 322mg (max. 2.54mmol) of crude liquid (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40***) were obtained.

3.1.2.2. Synthesis of (R,S)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (40**; 2nd trial)

1.23g (8.83mmol) of (*R,S*)-3,4-epoxybutyric acid (**39**), 907mg (7.56mmol) of $MgSO_4$ and 60.1mg (0.911mmol) of $K^{13}CN$ (97% of ^{13}C atoms) were stirred at room temperature for 4h, then 164mg (3.35mmol) of NaCN were added, the mixture was stirred for more 21h, acidified with conc. $HCl_{(aq)}$, and extracted 8 x 5.0ml of ethyl acetate. The combined organic layers were dried over Na_2SO_4, and drained. The solvent was evaporated. 754mg (max. 4.26mmol) of crude liquid (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40****) were obtained.

3.1.2.3. Synthesis of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*)

322mg (2.54mmol) of crude (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40***; obtained in the 1st trial) had been stirred in 15ml of the diazomethane (**42**)/diethyl ether solution at room temperature without stoppering, until the solvent evaporated. Then next 15ml of the diazomethane (**42**) solution were added, and reaction was continued as above. 15ml of 7M NH_3 in methanol were added to obtained crude methyl (*R,S*)-[5-^{13}C]-3-hydroxy-4-cyanobutyroate (**41***), and the mixture was stirred in a stoppered vial at room temperature for 99h. The solvent was evaporated at room temperature, and next 15ml of the ammonia solution were added, and the reaction was continued for more 109h in the similar manner. The residue obtained after the evaporation of the solvent was

loaded to a silica gel column, and eluted with acetone. 22.0mg (0.175mmol) of white solid (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***; 27.3 % of ^{13}C atoms; 24.8-fold isotopic enrichment) with 6.89% yield, and with 2.28% isotopic yield calculated from starting K^{13}CN.
^{1}H-NMR (300MHz, CD_3OD): δ 2.36-2.51 (2H, 2-CH_2, m), 2.53-2.76 (2H, 4-CH_2, m), 4.23-4.35 (1H, CH, m); ^{13}C-NMR (75 MHz, CD_3OD): δ 26.3 (C-4), 42.9 (C-2), 65.7 (C-3), 119.1 (CN), 175.6 ($CONH_2$)

3.2. Investigation of mechanism of racemisation of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) with NHase from *Rhodococcus erythropolis* NCIB 11450

This mechanism (scheme 18) was studied using previously synthesised (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***; 3.1.2.). Although it had been observed for 3-benzyloxy compound **34**, it was very probable it also should work with 3-hydroxy compound **37**.

3.2.1. Investigation of mechanism of racemisation of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) with cell free extract from *Rhodococcus erythropolis* NCIB 11450

The cell-free extract from Rhodococcus erythropolis NCIB 11450 contains NHase activity, but no amidase activity. Thus, the simplest way of investigating of the proposed mechanism of racemisation was to put **37*** in presence of cell-free extract, and check after some time whether some isotopic exchange of carbon atoms occurred between cyano and carbonyl groups of **37*** by comparing ^{13}C-NMR spectra.

It was performed this way (3.2.1.1.), the reaction was controlled after 22, 118 and 309h. The product was purified, and ^{13}C-NMR spectra were taken. Ratios of heights of peaks of cyano (119.1ppm), and carbonyl (175.6ppm) were observed. However, they did not change during reaction. If the predictions were true, we should have observed a very big increase (increasing with lengthening of the time of the reaction) of height of carbonyl peak, and decrease of cyano peak. The ratio of heights of these peaks should have been almost the same as in non-labeled **37**, if racemisation had occurred completely. However, it seemed it did not occur this way, since no such observation was done. The results are shown in the table 1. The maximal possible heights of carbonyl peaks are reported, since they are very small, not higher than the baselines.

It did not have to mean that the proposed mechanism was false. There are few reasons of

such a result, e.g.: the activity of NHase might have been to small to cause racemisation (but this amount converts 3-hydroxyglutarodinitrile (**44**) into **40**, therefore there should have been enough enzyme in the system); or NHase present in the cell-free extract was not the one, which causes the racemisation. It was necessary to perform an experiment with whole cells of *R. erythropolis* NCIB 11450, since this phenomenon was observed in the reaction catalysed by whole cells.

3.2.1.1. Investigation of mechanism of racemisation of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) with cell free extract from *Rhodococcus erythropolis* NCIB 11450

The mixture of 10.8g (84.4μmol) of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***) and 4.00ml of cell-free extract from *Rhodococcus erythropolis* NCIB 11450 in 20.0ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 ^{o}C. 3 equal volumes of solution were taken – after 22h, 118h and 309h, respectively. The following procedure was applied to each of these fractions. The solvent was evaporated. The residue was purified on a silica gel column using acetone as an eluent. The ^{13}C-NMR spectrum (75MHz, CD_3OD) of purified (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***) was recorded.

The results are shown in table 1, relative (the height of the nitrile carbon (5th position) peak (119.1ppm) equals 1.000) atom peak heights of peaks are presented.

Table 1. Results of investigation of mechanism of racemisation of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) catalyzed by Rhodococcus erythropolis NCIB 11450 cell-free extract.

	175.6ppm	119.1ppm	65.7ppm	42.9ppm	26.3ppm
37	0.491	1.000	3.21	2.53	4.49
37*	0.0198	1.000	0.159	0.115	0.198
22h product	< 0.0400	1.000	0.176	0.112	0.176
118h product	< 0.0345	1.000	0.138	0.129	0.172
309h product	< 0.0372	1.000	0.107	0.0992	0.149

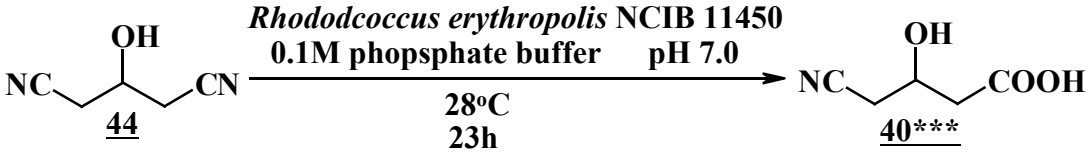
3.2.2. Investigation of mechanism of racemisation of (R,S)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) with *Rhodococcus erythropolis* NCIB 11450 whole cells

The experiment using the *Rhodococcus erythropolis* NCIB 11450 cell-free extract (3.2.1.) didn't show any racemisation of used (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***). However, the reason there might have been that the cell-free extract didn't contain NHase proper to catalyse the racemisation; it did not have to mean that this process occurs in a different way, than proposed

in our goup. Therefore, there was a need to perform this reaction with whole cells of used bacteria; especially because the phenomenon of the racemisation had been observed in the experiment with whole cells.

A complication here was the fact, that whole cells of these microorganisms convert **37** into **40**, since they contain an amidase activity, too. Therefore at first ^{13}C-NMR spectrum of higher amounts of **40** should be taken in order to know ^{13}C chemical shifts of this compound, and the synthesis of **40** was necessary.

The synthesis of **40***** (3.2.2.1.) was performed via the enzymatic hydrolysis of 3-hydroxyglutarodinitrile (**44**; previously obtained in our group by Christien Schortinghuis; scheme 21). It gave a very good yield; however, it was difficult to measure; the product after column separation was still crude. But the ^{13}C-NMR spectrum was good enough to know the shifts of **37*****.



Scheme 21. Synthesis of 3-hydroxy-4-cyanobutyric acid.

The idea of the studies on the mechanism of racemisation of **37*** catalyzed by *R. erythropolis* NCIB 11450 whole cells (3.2.2.2.) was almost the same, as in the case of investigating of the cell-free extract, and was described previously (3.2.1.).

The reaction was controlled after 17 and 198h, but it was performed differently than in the previous experiment. After 17 h there was not a lot of cyano-acid **40***.; there was mostly non converted cyano-amide **37*** (TLC). The obtained mixture was then reacted for longer time. After 198h there was only **40*** (TLC). ^{13}C-NMR spectra of 1st control (table 2) and 2nd control (table 3) were taken.

Similarly to the cell-free extract experiment, ratios of heights of cyano (119.3ppm for **40**) and carbonyl (174.7ppm for **40**) were the most important thing here.

The result of this experiment is also similar to the corresponding one of the cell-free extract. No isotopic exchange of carbon-13 between cyano and carbonyl groups was observed.

There are a few reasons of this fact, e.g. 1) this phenomenon does not occur for 3-hydroxy compound **37**, but it does for 3-benzyloxy **34**; therefore studies using **32** are necessary here; 2) investigating of isotopic exchange was not proper for these studies; 3) the mechanism of the investigated phenomenon occurred in different way than proposed.

The best solution here would be a use of similarly labelled chiral **34**. Using this compound, it would be possible to control, whether the racemisation occurs, and also to control the carbon exchange between the amido and cyano groups.

However; the studies performed here did not confirm the proposed mechanism of the decreasing of e.e. of hydrolysis of **36** to **35**.

3.2.2.1. Enzymatic hydrolysis of 3-hydroxyglutarodinitrile (44) with *Rhodococcus erythropolis* NCIB 11450 cells

The mixture of 234mg (2.15mmol) of 3-hydroxyglutarodinitrile (**44**) and 700mg of *Rhodococcus erythropolis* NCIB 11450 cells in 210ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 ^{o}C for 23 hours. Then the mixture was acidified with 5.0ml of conc. $HCl_{(aq)}$. The solvent was evaporated, and the obtained residue was purified on a silica gel column using ethyl acetate as an eluent. 445mg of impure liquid 3-hydroxy-4-cyanobutyric acid (**40*****) were obtained.

^{1}H-NMR (300MHz, DMSO-d_6): δ 1.91-2.10 (2H, 2-CH_2, m), 2.50-2.78 (2H, 4-CH_2, m), 4.25-4.35 (1H, CH, m), 8.35 (1H, COOH, bs); ^{13}C-NMR (75MHz, DMSO-d_6): δ 26.4 (C-4), 41.9 (C-2), 65.5 (C-3), 119.4 (CN), 174.7 (COOH)

3.2.2.2. Investigation of mechanism of racemisation of (*R,S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) with *Rhodococcus erythropolis* NCIB 11450 cells

The mixture of 11.2g (88.2μmol) of (R,S)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***) and 30mg of *Rhodococcus erythropolis* NCIB 11450 cells in 5.00ml of 0.1M sodium pyrophosphate buffer, pH 7.0, was shaken 200rpm at 28 ^{o}C for 17h. The solvent was evaporated. The residue was purified on a silica gel column using methanol as an eluent, and non-reacted substrate was recovered. It was dissolved in 5.00ml of of 0.1M sodium pyrophosphate buffer, pH 7.0, and 32mg of *Rhodococcus erythropolis* NCIB 11450 cells were added. The mixture was shaken 200rpm in 28 ^{o}C for 198h, and it was acidified with conc. $HCl_{(aq)}$. The solvent was evaporated, the residue was purified on a silica gel column using methanol as an eluent. The NMR spectra (300MHz, CD_3OD) of purified non-reacted 3-hydroxy-4-[5-^{13}C]-cyanobutyramide (**37***) and 3-hydroxy-4-[5-^{13}C]-cyanobutyric acid (**40***) after conversion, were taken.

The results are shown in tables 2 and 3 (for the amide and for the acid, respectively), relative (the height of nitrile carbon (5 position) peak (119.1ppm) equals to 1.000) peak heights are presented.

Table 2. Results of investigation of mechanism of racemisation of (*R*,*S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) catalyzed by Rhodococcus erythropolis NCIB 11450 whole cells – 1st control.

	175.6ppm	119.1ppm	65.7ppm	42.9ppm	26.3ppm
37	0.491	1.000	3.21	2.53	4.49
37*	0.0198	1.000	0.159	0.115	0.198
17h product – amide	< 0.0312	1.000	0.164	0.125	0.156

Table 3. Results of investigation of mechanism of racemisation of (*R*,*S*)-3-hydroxy-4-[5-^{13}C]-cyanobutyramide (37*) catalyzed by Rhodococcus erythropolis NCIB 11450 whole cells – 2nd control.

	174.7ppm	119.3ppm	65.4ppm	41.9ppm	26.3ppm
40***	1.58	1.000	6.06	4.22	5.89
198h product – acid	< 0.0625	1.000	0.133	0.141	0.164

4. Summary

In the presented work a few different subjects were studied.

Syntheses of 2,2-dicyanohexane (**1**; 2.1.1.) and 2-cyano-3-(*p*-hydroxyphenyl)-propionitrile (**13**; 2.2.1.) as substrates for enzymatic hydrolyses using *Rhodococcus erythropolis* NCIB 11450 cells or cell-free extract (2.1.2., 2.2.2.) were performed.

These substrates were used in the above mentioned hydrolyses. **1**, **13** and **26** (2.3.3.) gave corresponding cyano-amides 2-cyano2-methylhexanamide (**4**; 2.1.3.), 2-cyano -3-(*p*-hydroxyphenyl)-propionamide (**16**) and 2-cyano-3-(*p*-hydroxyphenyl)-propionamide (**28**) as the products of the hydrolyses. It was proven for the first time for hydrolyses of **1** and **13** using *R. erythropolis* NCIB 11450.

The racemic cyano-amides: **4** (2.1.3.), **16** (2.2.3.) and **28** (2.3.2.) – racemic products of the enzymatic hydrolyses were synthesised in order to find assays for determining e.e.s of enzymatic reactions. However, no proper method was found for **16** and **28**, although different HPLC and GC chiral columns were tested. Therefore the studies on e.e.s of those reactions weren't possible.

The syntheses of the mentioned substrates and products of the enzymatic hydrolyses were performed via the Knoevenagel condensations of the corresponding aldehydes with the cyano compounds, subsequent reductions of the obtained alk-2-enes to alkanes (which were often most limiting steps of syntheses, different reducing agents were necessary), and optional methylations to α-methyl compounds.

The cyano-amides obtained in the enzymatic hydrolyses of **13** and **26** were converted into tyrosines (**20**; 2.2.4.) or phenylalanines (**32**; 2.3.3.) via the Hoffman rearrangements in mild conditions, and subsequent hydrolyses into amino acids.

The similar Hoffman rearrangements were used for syntheses of racemic amino nitriles of tyrosine (**21**; 2.2.4.5.) and phenylalanine (**33**; 2.3.4.). The obtained nitriles were then resolved to the mentioned amino acids using *R. erythropolis* NCIB 11450 cells.

All these conversions of cyano-amides into amino acids were performed in order to find a novel way of synthesis of amino acids, and to determine e.e.s of enzymatic reactions indirectly (2.2.4.4.). However, they were not successful due to the very small yields in the Hoffman rearrangements, since iodosobenzene bis-(trifluoroacetate) **22** decomposed aromatic systems.

Moreover, the formation of amido-nitriles leading to D-amino acids, or these amino acids themself seem to be preferred during the mentioned enzymatic processes.

The other part of this work was devoted to the investigations of the proposed mechanism of the racemisation of 3-benzyloxy-5-cyanobutyramide (**34**) in the presence of NHase from *R. erythropolis* NCIB 11450. The formation of 6-membered ring was proposed. The investigations

were performed using non-benzylated analogue **37**.

At first the syntheses of (R,S)-3-hydroxy-5-cyanobutyramide (**37**; 3.1.1.), and then [5-^{13}C]-labelled **37*** (3.1.2.) were performed. Unfortunately, the synthesis of the labelled compound gave very small yield, the limiting step there was the incorporation of labelled cyanide.

Synthesised **37*** was used for the studies on the mechanism mentioned above. If the mechanism was true, isotopic exchange of label between the cyano and the carbonyl group should have been seen (using ^{13}C-NMR) after treatment of **37*** with NHase. However, no such an observation was made using the cell-free extract (3.2.1.) and whole cells (3.2.2.) of *R. erythropolis* NCIB 11450. Some studies using analogously labelled chiral **34** are necessary to confirm these conclusions.

The enzymatic hydrolyses of prochiral dinitriles are very attractive subjects for scientists. They are still unexplored and there are still a lot of interesting phenomena to be studied by organic chemists and biochemists.

5. References

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