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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 19 (2008) 562–567

Nitrilase-catalysed hydrolysis of cyanomethyl *p*-tolyl sulfoxide: stereochemistry and mechanism

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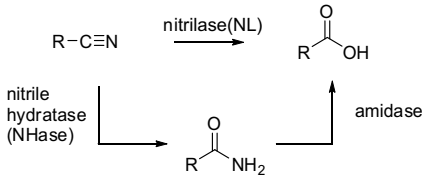
Received 14 December 2007; accepted 16 January 2008
Available online 7 February 2008

Abstract—Several commercially available nitrilases have been used for the enantioselective hydrolysis of cyanomethyl *p*-tolyl sulfoxide into the corresponding amide and acid, which are formed in different proportions and with varying stereoselectivities, depending on the nitrilase involved. It was shown that the externally added amide is not transformed into the acid, which can be explained by assuming that both products must be produced in concurrent reactions. It was also demonstrated that the absolute configuration of the substrate exerts substantial influence on the product ratio. Two alternative explanations of the stereochemical course are presented.

1. Introduction

Nitriles constitute as an important and versatile class of intermediates for synthetic chemistry.[1] They are synthetically particularly useful and, hence, widely used for the preparation of various carboxylic acids and amine derivatives. However, their chemical transformation into the corresponding carboxamides and carboxylic acids or esters usually requires the use of strong bases, acids or heavy metal salts. Therefore, an alternative approach to achieve this, which rests upon the use of nitrile hydrolysing enzymes, has been a fast developing area of research in recent years.[2–7]

It is generally accepted that the enzyme-catalysed hydrolysis of nitriles follows one of the two pathways (Scheme 1).[8] In the first pathway, a nitrilase (NL) converts the nitrile directly into the carboxylic acid, via addition of two molecules of water. In the second one, a nitrile hydratase (NHase) catalyses single hydration of the nitrile to give the corresponding amide, which is followed by amidase-catalysed hydrolysis to the appropriate acid. Thus, according to this scheme, the latter pathway requires the simultaneous presence of two different enzymes, which can be achieved using microorganisms or whole cell techniques.



Scheme 1. Pathways of enzyme-catalysed nitrile hydrolysis.

This simple picture has recently turned out to be insufficient for explaining new findings. For example, it was reported that certain nitrilases, besides their anticipated behaviour, exhibited NHase activity, which resulted in the formation of both the corresponding amides and acids.[9–15] In principle, such results could be explained by invoking the second pathway. However, on the basis of common observations that nitrilases do not hydrolyse carboxamides,[13] the assumption was made that both products of the hydrolysis were formed in parallel reactions. A plausible mechanism, which could account for such results, was proposed by Sheldon et al.[13]

Our previous work on the deracemisation of racemic and desymmetrisation of prochiral heteroorganic compounds led to efficient approaches for the synthesis of a variety

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doi:10.1016/j.tetasy.2008.01.021

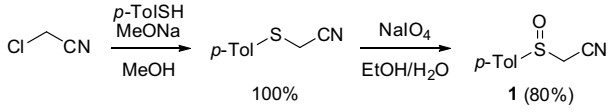
of optically active heteroatom derivatives.[14–18] In particular, our results on the enzymatic desymmetrisation of bis(cyanomethyl) sulfoxide[14] and bis(cyanomethyl)phenylphosphine oxide[15] represented the first examples of a nitrilase-catalysed stereoselective hydrolysis of dinitriles containing prostereogenic centres located on a heteroatom—the sulfinyl sulfur and phosphinyl phosphorus, respectively. In these cases, the hydrolysis also led to two out of five possible products, namely the corresponding monoamide and monoacid, formed in different proportions and with varying enantiomeric excesses, depending on the kind of the nitrilase. Interestingly, in certain cases the absolute configurations of the amide and acid produced in the same reaction were identical, while in others they were opposite. The former would substantiate the assumption that both products were formed in a concurrent reaction (as proposed by Sheldon et al.),[13] while the latter would suggest that the amide was first formed non-stereoselectively and then stereoselectively hydrolysed to the acid under kinetically controlled resolution conditions.

The use of prochiral substrates did not allow us to distinguish the two possible pathways employed by the enzymes. First of all, it was not possible to determine whether the first step leading to the amide was stereoselective, since the unreacted substrate was not chiral. Additionally, it appeared rather difficult to synthesise a racemic monoamide for subjection to hydrolysis by a nitrilase. In order to gain better insight into the hydrolysis mechanism, we decided to investigate the nitrilase-catalysed kinetic resolution of a racemic cyanomethyl sulfoxide since this would provide more information about the stereochemistry of the reaction. As a model substrate, the easily available racemic cyanomethyl *p*-tolyl sulfoxide **1** was chosen.

2. Results and discussion

2.1. Synthesis of substrates

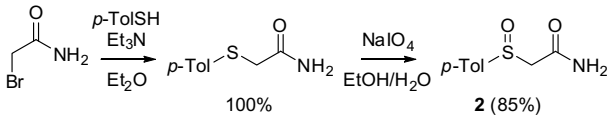
2.1.1. Synthesis of racemic and enantiomeric cyanomethyl *p*-tolyl sulfoxides. The starting material, racemic cyanomethyl *p*-tolyl sulfoxide, **1**, was synthesised in a two-step procedure starting from chloroacetonitrile, which on subjection to *p*-toluenethiol in the presence of sodium methoxide in methanol provided cyanomethyl *p*-tolyl sulfide in quantitative yield. The latter was oxidised with sodium periodate in a 1:1 mixture of water and ethanol to give the desired sulfoxide **1** in 80% yield after purification (Scheme 2).



Scheme 2. Synthesis of racemic sulfoxide **1**.

The enantiopure compounds (+)-(*R*)-**1** and (−)-(*S*)-**1** were synthesised using a method described by Hiroi and Umemura.[19]

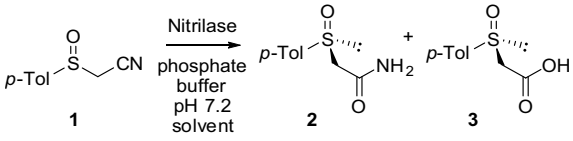
2.1.2. Synthesis of racemic *p*-toluenesulfinylacetamide 2. Racemic **2** was synthesised via a two-step method from bromoacetamide, which upon treatment with *p*-toluenethiol in the presence of triethylamine in Et_2O, provided the corresponding sulfide in quantitative yield. The latter was oxidised with sodium periodate in a 1:1 mixture of water and ethanol to give the desired racemic amide **2** in 85% yield after purification (Scheme 3).



Scheme 3. Synthesis of racemic amide **2**.

2.2. Kinetic resolution of racemic cyanomethyl *p*-tolyl sulfoxide 1

The racemic sulfoxide **1** was subjected to hydrolysis in a phosphate buffer and a co-solvent to dissolve the substrate, by a variety of nitrilases under kinetic resolution conditions. The hydrolysis should in principle give rise to two products: *p*-toluenesulfinylacetamide **2** and *p*-toluenesulfinylacetic acid **3**, together with (probably) enantiomerically enriched starting material **1** (Scheme 4, absolute configurations of **2** and **3** are arbitrarily chosen). The products were purified by column chromatography and analysed spectroscopically. The results are shown in Table 1.



Scheme 4. Enzymatic hydrolysis of sulfoxide **1**.

The enantiomeric excesses of acid **3** were calculated by comparing the $[\alpha]_D$ value of a given sample with that reported in the chemical literature,[20] while the enantiomeric excesses of unreacted nitrile **1** and amide **2** were determined by chiral HPLC. The absolute configurations of **1** and **3** are known from the literature to be (+)-(*R*).[19,20] To determine the absolute configuration of amide **2**, CD spectra of (+)-(*R*)-**1**, (+)-(*R*)-**3** and of (+)- and (−)-**2** were measured as shown in Figure 1.

It is justifiable to assume that the comparison of the Cotton effects exhibited by each sample allows to ascribe an absolute configuration to amide **2**, since the stereogenic sulfur atom in all the compounds is bound to three identical substituents, and the fourth is only slightly different at a distant position. As can be seen in Figure 1, the Cotton effects exhibited by the laevorotatory amide (−)-**2** and the laevorotatory acid (−)-(*S*)-**3** are almost identical. Furthermore, the Cotton effects exhibited by the dextrorotatory amide (+)-**2** and the dextrorotatory nitrile (+)-(*R*)-**1** are identical as well. Hence, it seems reasonable to assign the absolute configuration of amide **2** as being (+)-(*R*).

Table 1. Nitrilase-promoted hydrolysis of cyanomethyl *p*-tolyl sulfoxide

Entry	Nitrilase	Solvent	Time (h)	Product; yield (%)	$[\alpha]_D$	ee (%)	Absolute configuration
1	NL 103	$CHCl_3$	120	**1**; 40.0	+42.5[a]	37[c]	(*R*)
				2; 30.0	−51.5[b]	21[c]	(*S*)
				3; 30.0	−63.8[b]	35[d]	(*S*)
2	NL 103	Acetone	120	**1**; 46.0	+38.0[a]	33[c]	(*R*)
				2; 27.0	−47.0[b]	20[c]	(*S*)
				3; 19.0	−56.0[b]	31[d]	(*S*)
3	NL 104	$CHCl_3$	72	**1**; 10.2	+50.1[a]	44[c]	(*R*)
				2; 32.1	+171.2[b]	71[c]	(*R*)
				3; 57.1	−48.8[b]	27[d]	(*S*)
4	NL 104	Acetone	72	**1**; 26.0	+29.8[a]	26[c]	(*R*)
				2; 33.0	+150.0[b]	62[c]	(*R*)
				3; 36.4	−43.0[b]	24[d]	(*S*)
5	NL 105	$CHCl_3$	120	**1**; 72.4	0[a]	0	—
				3; 25.3	−2.0[b]	1[d]	(*S*)
6	NL 105	Acetone	120	**1**; 80.0	0[a]	0	—
				3; 9.1	−1.6[b]	1[d]	(*S*)
7	NL 106	$CHCl_3$	72	**2**; 10.0	+90.0[b]	37[c]	(*R*)
				3; 90.0	−13.4[b]	7[d]	(*S*)
8	NL 107	$CHCl_3$	72	**2**; 36.0	+31.5[b]	13[c]	(*R*)
				3; 60.1	−30.2[b]	17[d]	(*S*)
9	NL 108	$CHCl_3$	120	**1**; 62.8	0[a]	0	—
				3; 30.5	−3.8[b]	2[d]	(*S*)
10	NL 110	$CHCl_3$	120	**1**; 100	0[a]	0	—
11	NL 111	$CHCl_3$	120	**1**; 100	0[a]	0	—
12	NL 112	$CHCl_3$	120	**2**; 62.0	+109.0[b]	45[c]	(*R*)
				3; 38.0	−149.1[b]	83[d]	(*S*)
13	NL 112	Acetone	120	**2**; 34.5	+100.0[b]	42[c]	(*R*)
				3; 58.2	−137.5[b]	76[d]	(*S*)

[a] In $CHCl_3$ (*c* 1).
[b] In MeOH (*c* 1).
[c] Determined by chiral HPLC.
[d] Enantiomeric excess, as determined by comparison of $[\alpha]_D$ values.

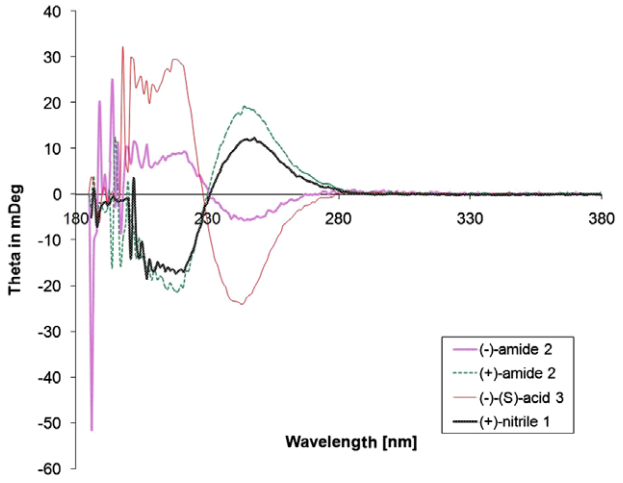


Figure 1. The CD spectra of compounds **1**, **2** and **3**.

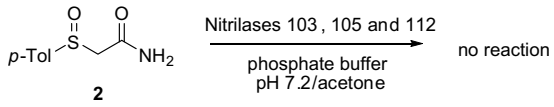
Correctness of this assignment was ultimately confirmed by chemical, acid-catalysed hydrolysis of (+)-amide **2**, which gave acid (+)-(*R*)-**3**. Since the transformation took place beyond the stereogenic sulfur atom, the absolute configuration of the amide must be (+)-(*R*)-**2**.

Inspection of Table 1 clearly shows that in most cases, the reactions were not selective and result in the formation of both nitrile hydrolysis products, that is, amide **2** and acid **3**. No substantial differences were observed with the change of a co-solvent from chloroform (a biphasic system) to acetone (homogeneous solution). Interestingly, nitrilases NL 110 and NL 111 (entries 10 and 11) turned out to be completely unreactive under the conditions applied. In two cases, it was possible to recover the enantiomerically enriched unreacted substrate **1** (entries 1 and 4), while in three other cases, the isolated unreacted substrate was racemic (entries 5, 6 and 9). The stereoselectivity of the hydrolysis was generally lower as compared to the desymmetrisations that we previously described.[14,15] Nevertheless, the stereochemical outcome of particular experiments allowed us to draw some conclusions concerning the reaction mechanism. To start with, the reaction in which the absolute configuration of both products, amide **2** and acid **3**, were the same (entries 1 and 2) may be taken as proof for the bidirectional mechanism in which both products are formed concurrently.[13] On the other hand, the cases in which the absolute configurations of the resulting amide and acid are opposite (entries 7, 8, 12 and 13) point to the pathway in which the amide is formed first and then subsequently hydrolysed. However, the assumption that the real stereorecognition is the result of a kinetic resolution of the initially formed racemic amide must be discarded since the isolated unreacted substrate was enantiomerically enriched (entries 1–4). In this context, it should be stressed that the three examples in which the recovered substrate is racemic

(entries 5, 6 and 9) show the exclusive formation of the acid in low yield and with very low enantiomeric excess.

2.3. Attempted nitrilase-catalysed hydrolysis of the racemic amide 2

To finally clarify the issue of the involvement of amide **2** as an intermediate in the formation of acid **3**, the racemic amide was synthesised and subjected to hydrolysis by selected nitrilases in order to investigate the formation of all possible reaction products (Scheme 5).



Scheme 5. Attempt to perform a nitrilase-catalysed hydrolysis of amide **2**.

Surprisingly, in none of these cases was the formation of acid **3** observed. This seems obvious for NL 103, which catalyses the nitrile hydrolysis to form both products **2** and **3** having the same absolute configuration (*S*), and to leave the non-racemic unreacted substrate **1** of opposite absolute (*R*)-configuration (entries 1 and 2). Hence, it is logical to assume that this nitrilase must employ the bidirectional mechanistic pathway. However, in the case of NL 112, which leads to the formation of both products having opposite absolute configurations (entries 12 and 13), this additional experiment suggests that the amide is not involved in the formation of the acid. Finally, in the case of NL 105 (entries 5 and 6), the result shown in Scheme 5 led us to discard the possible explanation that the amide is non-stereoselectively formed in a rate-determining step and then fully hydrolysed to the acid. On the other hand, the observed lack of reactivity of amides may only prove that the externally added amide cannot enter the active site. However, as suggested by a Referee,[21] if the amide was generated within the active site by hydrolysis of the nitrile, it could still react and this would be compatible with our findings (see the discussion in Section 3).

2.4. Nitrilase-catalysed hydrolysis of the enantiomers of 1

To determine the relationship between the absolute configuration of substrate **1** and the ratio of the particular hydrolysis products **2** and **3**, each enantiomer of **1** was separately subjected to hydrolysis by selected nitrilases. The results are shown in Table 2.

Inspection of Table 2 reveals a tendency towards the preferential formation of amide **2** from (+)-(*R*)-**1** and of acid **3** from (−)-(*S*)-**1** for NL 103 (entries 1 and 2) and NL 112 (entries 3 and 4). Although the relationship is not so clear-cut, probably due to the relatively low stereoselectivity of the hydrolysis (cf. Table 1, entries 1 and 12), it shows that the absolute configuration of the substrate has a substantial influence on the reaction course.

3. Mechanism of action

There is no X-ray analysis of nitrilases available so that the actual structure of their active sites remain unknown. However, it is generally accepted that cysteine, which is part of the catalytic triad Cys-Glu-Lys, plays the role of the reactive amino acid. The sulfhydryl group of cysteine is believed to attack the nitrile carbon atom in the first step to form a thioimidate intermediate. The bidirectional mechanism of nitrile hydrolysis proposed by Sheldon et al.[13] assumes that the thioimidate is the common primary intermediate for both hydrolysis pathways. Once formed, it can be transformed in two ways: it either reacts with a single water molecule to form an amide, or it reacts with two water molecules to provide an acid. This mechanism also predicts that transformation of the amide into the corresponding acid is energetically unfavourable.

The aforementioned results strongly favour this mechanism, particularly if one takes into account our findings that the externally added amide was in none of these cases transformed into the acid. However, this mechanism cannot account for all the results obtained. First of all, it is not applicable to all nitrilases used in these investigations, since the hydrolysis results were in some cases entirely different. Secondly, it does not explain the stereochemistry of the reaction. The case in which both the amide and the acid formed have the same and the recovered unreacted substrate the opposite absolute configurations may be taken as proof that both reactions take place within the same chiral environment influencing stereorecognition. On the other hand, the results in which the amide and the acid formed have opposite absolute configurations lead to an assumption that each reaction may employ different pockets of the enzyme active site at the enantioselective water

Table 2. Nitrilase-promoted hydrolysis of enantiomers of cyanomethyl *p*-tolyl sulfoxide **1**

Entry	Nitrilase	Substrate	Time (h)	Product; yield (%)	$[\alpha]_D$	ee (%)	Absolute configuration
1	NL 103	(+)-**1** ee >95%	120	**1**; 78.0	+115	>99	(*R*)
				2; 14.0	+180	75	(*R*)
				3; 8.0	+7.2	4	(*R*)
2	NL 103	(−)-**1** ee = 75%	120	**2**; 11.0	−230	95	(*S*)
				3; 86.0	−120	66	(*S*)
3	NL 112	(+)-**1** ee >95%	72	**2**; 86.0	+201	83	(*R*)
				3; 13.0	+18	10	(*R*)
4	NL 112	(−)-**1** ee = 75%	72	**2**; 20.0	−160	66	(*S*)
				3; 75.0	−100	55	(*S*)

attack on the preliminary thioimidate intermediate. The fact that each enantiomer of the substrate produces preferentially one of the hydrolysis products may support this assumption.

For the sake of the completeness of the discussion, another possible explanation of the stereochemical course of the reaction investigated can be taken into account.[21] Thus, it can be assumed that the nitrile is first converted into the amide. The active site may be large enough to enable the amide to rotate within it. For the nitrile, the sulfoxide oxygen is likely to H-bond to a residue in the active site, but for the amide it may be the carbonyl which for some enzymes selectively H-bonds to that residue, thus inverting the enantioselectivity of the hydrolysis to the acid. For other enzyme/substrate combinations, such a rotation may not be possible and so both hydrolyses occur with the same enantioselectivity. In both cases, the amide may be expelled from the active site at a rate commensurate with its subsequent hydrolysis and, if electrostatic effects prevent its re-entry to the active site, this would explain the fact that both the amide and the acid are obtained and the external amide does not undergo hydrolysis.

4. Conclusions

We have shown that a variety of nitrilases convert cyanomethyl *p*-tolyl sulfoxide **1** into the corresponding amide and acid, which are formed in various proportions and with various stereoselectivities, depending on the nitrilase involved. We have proven that the externally added amide is not transformed into the acid, which suggests that both products may be produced in concurrent reactions. We have found that the absolute configuration of the substrate exerts substantial influence on the ratio of products. On this basis, two alternative explanations of the stereochemical course of the reaction have been presented. Although most of the results obtained by us are in agreement with the bidirectional mechanism proposed by Sheldon et al.,[13] some of them clearly demonstrate that the Sheldon mechanism is incapable of accommodating all reactions outcomes. Possibly, better explanations have to await determination of crystal structures of nitrilases, which so far do not exist.

5. Experimental

5.1. General

The enzymes were purchased from BioCatalytic Europe GmbH, Grambach, Austria. NMR spectra were recorded on Bruker instruments at 200 MHz with $CDCl_3$, CD_3CN and CD_3OD as solvents. Optical rotations were measured on a Perkin–Elmer 241 MC polarimeter (*c* 1). Column chromatography was carried out using Merck 60 silica gel. TLC was performed on Merck 60 F_{254} silica gel plates. The enantiomeric excess (ee) values were determined by chiral HPLC (Varian Pro Star 210, Chiralpak AS).

5.2. Synthesis of racemic cyanomethyl *p*-tolyl sulfoxide 1

To a solution of sodium methoxide, prepared from methanol (30 mL) and sodium (1.86 g, 0.081 mol) a solution of *p*-toluenethiol (10 g, 0.081 mol) in methanol (100 mL) was added, followed by chloroacetonitrile (6.12 g, 0.081 mol). The mixture was stirred at room temperature for 1 h. After this time TLC revealed only one spot. Methanol was evaporated, the residue was treated with water and extracted with chloroform. After drying over anhydrous $MgSO_4$ and evaporation of $CHCl_3$ cyanomethyl *p*-tolyl sulfide was obtained as yellow crystals (13.14 g, 100%); ^{1}H NMR ($CDCl_3$): $\delta = 2.42$ (s, 3H), 3.55 (s, 2H), 7.38–7.62 (m, 4H).

The sulfide (13.1 g, 0.081 mol) was dissolved in an ethanol/water mixture (1:1, 200 mL), then $NaIO_4$ (34 g, 0.162 mol) was added and the mixture was stirred at room temperature until TLC revealed completion of the reaction (ca. 12 h). A white precipitate was filtered off, ethanol was evaporated and the aqueous residue was extracted with chloroform. After drying of the combined organic layers over anhydrous $MgSO_4$ and evaporation of chloroform, the residue was purified by column chromatography ($CHCl_3$) to afford **1** as a white powder (11.54 g, 80%); mp 60–62 °C; ^{1}H NMR ($CDCl_3$): $\delta = 2.42$ (s, 3H), 3.74 (AB system, 2H), 7.38–7.62 (m, 4H); ^{13}C NMR ($CDCl_3$): $\delta = 21.36$, 44.53, 111.19, 123.97, 129.94, 130.18, 138.03, 143.31; MS (CI): *m*/*z* 180 (M+H). Anal. Calcd for C_9H_9NOS: C, 60.31; H, 5.06; N, 7.81. Found: C, 60.15; H, 4.91; N, 7.75.

5.3. Synthesis of racemic *p*-toluenesulfinylacetamide 2

p-Toluenethiol (1.86 g, 0.015 mol) and bromoacetamide 2.07 g (0.015 mol) were dissolved in Et_2O (40 mL), and triethylamine (2.09 mL, 0.015 mol) in Et_2O (10 mL) was added slowly to the solution. The mixture was stirred for 1 h at room temperature and monitored by TLC. After completion of the reaction, Et_2O was evaporated, the residue was treated with water and extracted with CH_2Cl_2. After drying of the organic solution over anhydrous $MgSO_4$ and evaporation of dichloromethane, the corresponding sulfide was obtained as a white powder (2.72 g, 100%). The crude sulfide (TLC pure) was oxidised as above and purified by column chromatography ($CHCl_3$/MeOH from 100:1 to 25:1) to afford racemic **2** as a white powder (2.52 g, 85%); mp 141–143 °C; ^{1}H NMR (CD_3CN): $\delta = 2.40$ (s, 3H), 3.58 (AB system, 2H), 5.88 (br s, 1H), 6.50 (br s, 1H), 7.38–7.55 (m, 4H); ^{13}C NMR (CD_3CN): $\delta = 21.40$, 47.50, 125.58, 131.21, 140.39, 143.92, 170.29; MS (CI): *m*/*z* 198 (M+H). Anal. Calcd for $C_9H_{11}NO_2S$: C, 54.80; H, 5.62; N, 7.10. Found: C, 54.62; H, 5.70; N, 7.03.

5.4. Nitrilase-catalysed kinetic resolution of 1—general procedure

Cyanomethyl *p*-tolyl sulfoxide **1** (racemic or optically active) (0.100 g, 0.56 mmol) was suspended in a phosphate buffer solution (pH 7.2) and a co-solvent (see Tables) was added. After addition of an enzyme (10 mg) the mixture

was shaken at 30 °C for 72–120 h (see Tables) and monitored by TLC. Then, water was evaporated and the residue was separated by column chromatography ($CHCl_3$/methanol in gradient) or preparative TLC ($CHCl_3$/methanol 1:1) to yield the corresponding products **2** and **3** and the unreacted substrate **1**. The yields and specific rotations are shown in Tables 1 and 2.

5.4.1. *p*-Toluenesulfinylacetic acid 3. Yellow crystals, mp 85–95 °C; ^{1}H NMR (CD_3OD): $\delta = 2.41$ (s, 3H), 4.85 (s, 2H), 7.39–7.64 (m, 4H); ^{13}C NMR (CD_3OD): $\delta = 21.39$, 64.1, 125.69, 131.18, 140.38, 143.56, 172.17; MS (CI): *m/z* 199 (M+H), 154 (M−CO_2). Anal. Calcd for $C_9H_{10}O_3S$: C, 54.54; H, 5.05. Found: C, 54.32; H, 4.97.

Acknowledgements

This work was carried out under a Polish-Dutch Joint Research Project: 'Dynamic kinetic resolution and desymmetrisation of sulfur and phosphorus compounds using enzymes'. Financial support from the Ministry of Science and Information Society Technologies (Poland), Grant No. 3 T09A 166 27 for P.K., is gratefully acknowledged.

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21. We thank a Referee for this suggestion.