Bacterial class A acid phosphatases as versatile tools in organic synthesis
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Chapter 5

Enantiomeric differentiation by the acid phosphatases from *Shigella flexneri* and *Salmonella enterica*; enzymatic phosphorylation and dephosphorylation as tools for preparative resolutions

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Abstract
The enantiomeric differentiation in the hydrolysis and synthesis of phosphomonoesters by the acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* ser. *typhimurium* LT2 (PhoN-Se) was investigated. PhoN-Se showed acceptable hydrolysis rates towards both *O*-phospho-DL-threonine and *O*-phospho-DL-serine. With *O*-phospho-DL-threonine initially optically pure (100% ee) L-threonine was formed. Only when 60% of the L-isomer was converted, D-threonine was formed. When *O*-phospho-DL-serine was hydrolyzed by PhoN-Se the D-isomer was preferred, but the ee values rapidly dropped to 50%. We conclude that enantioselectivity of PhoN-Se with threonine is mainly, but not solely determined by the β-carbon in threonine. The enzyme PhoN-Sf converted *O*-phospho-DL-serine with low enantioselectivity and was virtually inactive towards *O*-phospho-DL-threonine. This latter enzyme discriminates between the *trans-* and *cis-*isomers of 2-methylcyclohexanol phosphate, being two times more selective towards the *cis-*isomer.

Introduction
Configurational isomery (chirality and *cis*-trans isomery) is a key factor in the efficacy of many drug products, agrochemicals and food and flavour compounds. In most cases, only one optically active form is biologically active and racemic compounds are by definition saddled with 50% of chemical ballast. Thus the production of enantiomerically pure amino acids, amino alcohols, amines, alcohols, and epoxides has become increasingly important. For the synthesis of these intermediates, the chemical industry has primarily relied upon established chemical methods. However, biocatalysis is now accepted as one of the key methodologies for the preparation of chiral organic compounds.\[1-4\] The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often environmentally friendly and have a high chemo-, regio- and enantioselectivity. Their mild reaction conditions allow complex target compounds with multi-functional groups to be resolved effectively. Certain enzymes such as lipases and esterases are now considered as standard and routine reagents in organic synthesis and can be acquired and used as any other chemical for resolution through enantioselective hydrolysis, esterification or acylation. A number of methods have been developed for the asymmetric syntheses of amino acids but chemical synthesis followed by enzymatic resolution is still the preferred way.\[5-9\] The enzymatic resolution involves the use of hydrolytic enzymes which selectively degrade one isomer in the mixture, followed by separation.

Scheme 1. Resolution of DL-threonine by acid phosphatase.

Converting (amino) alcohols to their *O*-phosphate instead of carboxyl esters is advantageous because chemical phosphorylation, e.g. by POCl₃, polyphosphoric acid, or P₂O₅, modifies selectively the hydroxyl but not the amino groups, as does acylation. Further the phosphate moiety drastically changes the solubility of an amino alcohol and therefore separation of the compounds after resolution will be easier. It has been shown that it is indeed possible to resolve *O*-phospho-DL-threonine using acid phosphatases from wheat germ and potato (scheme 1) that prefer hydrolysis of *O*-phospho-L-threonine.\[8,10\] To broaden the application range of phosphatases, we investigated two non-specific acid phosphatases (NSAPs) that are structurally unrelated to the acid phosphatases from wheat.
germ and potato in their potential enantiomeric differentiation. NSAPs are a group of secreted bacterial enzymes with a broad substrate specificity that shows hydrolytic activity towards several different organic phosphate monoesters such as nucleotides and sugar phosphates.\textsuperscript{[11,12]} These enzymes are also capable of transferring a phosphate group from a donor (phosphomonoester and pyrophosphate) to a wide range of acceptors (alcohols).\textsuperscript{[13,14]} The acid phosphatase from \textit{Shigella flexneri} YSH 6000\textsuperscript{[15]} (PhoN-Sf) shows enantioselectivity in the phosphorylation of D- and L-glucose by pyrophosphate having a higher affinity for D-glucose.\textsuperscript{[13]} Furthermore, when vanadate is present in the active site, PhoN-Sf and PhoN-Se (the acid phosphatase from \textit{Salmonella enterica} ser. \textit{typhimurium} LT2)\textsuperscript{[16]} are able to carry out an enantioselective sulfoxidation with opposite preferences.\textsuperscript{[17]} This prompted us to investigate the possible enantiomeric differentiation of PhoN-Sf and PhoN-Se in synthesis and hydrolysis of phosphomonoesters.

\textbf{Results and discussion}

\textit{Dephosphorylation of O-phosphorylated amino acids.}

The acid phosphatases from \textit{Shigella flexneri} (PhoN-Sf) and \textit{Salmonella enterica} (PhoN-Se) were investigated in their ability to deracemize racemic alcohol mixtures. Hereeto, O-phosphorylated DL-threonine and -serine were chosen as substrates (Chart 1). Although threonine contains 2 stereocenters and thus 4 possible stereoisomers exist, the \textit{allo}threonine forms (2\textit{R},3\textit{R} and 2\textit{S},3\textit{S}) are not present in the reaction mixtures, so only 2 stereoisomers, 2\textit{R},3\textit{S} and 2\textit{S},3\textit{R}, are present, which represent D- and L-threonine respectively.

PhoN-Sf was virtually unable to dephosphorylate O-phospho-DL-threonine as shown by a very low conversion of 0.3\% after 5.5 h at pH 5.0 (Table 1, Figure 1). The observed ee value of 100\% in favour of the L-isomer is therefore not representative for the whole reaction. O-phospho-DL-serine conversion by PhoN-Sf after 24 hours at pH 5.0 was 10\% and at this low conversion, the ee value had already dropped to 13.7\% in favour of the D-isomer.

\textbf{Chart 1.} Enantiomers of O-phosphothreonine and O-phosphoserine.
Table 1. Conversions and ee values of dephosphorylation of O-phospho-DL-threonine and O-phospho-DL-serine by PhoN-Se and PhoN-Sf\[a].

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<td>pH 6.1</td>
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<td>Serine</td>
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\[a\] 5 μM PhoN and 100 mM substrate in 100 mM acetate buffer at 30°C

However, PhoN-Se showed reasonable reaction rates in dephosphorylation of O-phospho-DL-threonine and O-phospho-DL-serine (Figure 1). Therefore the pH dependency of the enantioselectivity of this enzyme was investigated in more detail (Table 1). Hydrolysis rates are not affected to a large extent between pH 3.9 and 5.0, but they clearly diminish at pH 6.25 and no activity is found at pH 3.3. To our surprise, only L-threonine was detected within 5 hours of incubation for pH values between 3.9 and 5.0 (~32% conversion) and O-phospho-D-threonine was not hydrolyzed, giving an ee value of 100%\[a]. The E-value of this reaction, calculated from conversion and ee of the product, is >200, which means that this dephosphorylation reaction can be used for preparative deracemization. In principle, the remaining O-phospho-D-threonine can be hydrolyzed and subjected to a threonine racemase\[18,19\] and phosphorylated again for further resolution. For O-phospho-DL-serine, the rate of the pH dependent dephosphorylation was equal to that seen with O-phospho-DL-threonine. However, already after 0.5 h of incubation the ee values were significantly lower (Figure 1) and further slowly decreased to about 50% after 24 hours. The dephosphorylation rate of O-phospho-D-serine was faster compared to its L-counterpart. This results in an E-value of 3.7 for the hydrolysis of O-phospho-DL-serine. Thus, PhoN-Se shows a slight preference for the hydrolysis of the D-isomer of serine, but surprisingly a high preference for the L-isomer of threonine. The fact that PhoN-Se shows significant enantioselectivity in the dephosphorylation of O-phospho-DL-threonine but much less with O-phospho-DL-serine indicates that PhoN-Se discriminates between the D- and L-counterparts mainly on basis of the configuration of the β-carbon adjacent to the OH group, and not the α-carbon adjacent to the NH₂ group. The extra methyl group in threonine forces the enzyme to recognize the L-form of O-phospho-DL-threonine. When the methyl group is absent as in serine, the D-form is preferred. The same selectivity has been observed for wheat germ acid phosphatase.\[8\]

Since it is known that the ee of an enantioselective reaction may strongly depend on the temperature, the temperature dependency of the O-phospho-DL-threonine dephosphorylation by PhoN-Se was investigated (T = 5-75°C). However, no effect was seen on the enantioselectivity of the reaction.

\[a\] The detection limit of D-threonine in this system is 0.75 mM. In this case, no D-threonine detected means that less than 1 mM of D-threonine is formed. This leads to an ee value of > 97% at 30% conversion after 5 h of reaction time.
Earlier it was observed that PhoN-Sf is able to phosphorylate primary alcohols to a much higher extent than secondary alcohols and that PhoN-Se phosphorylates both primary and secondary alcohols.\cite{13,14} Thus PhoN-Se appears to have a broader substrate specificity as seems also to be the case for the dephosphorylations as shown above with $O$-phospho-DL-serine and $O$-phospho-DL-threonine.

![Figure 1](image-url)  
\textbf{Figure 1.} Time course of the dephosphorylation of 100 mM $O$-phospho-DL-threonine (squares) and $O$-phospho-DL-serine (circles) by 5 \mu M PhoN-Sf (closed symbols) and PhoN-Se (open symbols) at pH 5.0; Panel A: Time course of the reaction. Panel B: ee values of the reaction products.

Virtually all enantioselectivities of enzymes originates from the energy difference in the enzyme-transition-state complex. Due to the chiral environment of the active site of the enzyme, diastereomeric enzyme-substrate complexes are formed, which possess different values of free energy for their respective transition states. The result is a difference in activation energy for both of the enantiomeric substrates. As a consequence, one enantiomer will be transformed faster than the other. The value of this difference in free energy is a direct measure for the selectivity of the reaction. To obtain structural insights in the factors that affect hydrolysis by PhoN-Se, docking of the phosphate esters of serine and threonine (both D and L) in the active site of PhoN-Se was performed. Since a 3D structure of PhoN-Se used in our study has not been reported, a model was generated using the recently resolved X-ray structure of \textit{Salmonella enterica} ser. \textit{typhimurium} MD6001 acid phosphatase (EMBL:AF521595,\cite{20} PDB code 2A96\cite{21}), showing 94\% amino acid sequence identity to PhoN-Se. Docking of the two enantiomers of $O$-phosphothreonine revealed a difference in binding energy of 3.21 kJ/mol in favour of the D-enantiomer. For the enantiomers of $O$-phosphoserine a difference of 0.69 kJ/mol was calculated in favour of the L-enantiomer. The calculated differences in free energy predict preferences which are not in agreement with the experimental data. However, the MD6001 phosphatase was co-crystallized with phosphate which was not covalently bound to the enzyme and therefore the structure does not represent the active enzyme-phosphate intermediate present during catalysis. The X-ray structure of the \textit{Eschericia blattae} acid phosphatase\cite{22} (EMBL accession no. AB020481, PDB code 1EO1), showing 38\% amino acid sequence identity with PhoN-Se was used to generate another model of PhoN-Se. Although the overall identity is much lower, the active site is pretty much conserved as depicted in Figure 2 showing the active site residues of the two PhoN-Se models generated. The two models only start to show larger differences outside 6Å from the phosphate and molybdate anions. Since the \textit{E. blattae} phosphatase was co-crystallized with molybdate covalently bound to NE2 of His 197, this is a more realistic model of the enzyme during catalysis. Docking of the two enantiomers of $O$-phosphothreonine revealed a difference in binding energy of 0.68 kJ/mol in favour of the L-enantiomer. For the enantiomers of $O$-phosphoserine a difference
of 1.27 kJ/mol was calculated in favour of the D-enantiomer. Using this model, the preference for the enantiomers is predicted correctly. It should be mentioned that docking of the ligands in the positions giving a low energy situation shows large flexibility. The phosphate group and the carbon atom to which the phosphate is esterified are placed consistently by the docking program, but the rest of the molecules show up on different positions. This is in agreement with the knowledge that this is a non-specific acid phosphatase that accepts a wide range of substrates.[11]

![Image of active site residues](Figure 2. Active site residues of the two PhoN-Se models based on the *Salmonella enterica* ser. *typhimurium* MD6001 and *Escherichia blattae* acid phosphatases with the co-crystallized phosphate and molybdate (covalently bound), respectively. Molecular graphics created with YASARA (www.yasara.org) and PovRay (www.povray.org)).

**Enantioselectivity in phosphorylation of racemates**

Many carbohydrates and alcohols can be phosphorylated by PhoN-Sf and PhoN-Se with pyrophosphate (PPi) as a phosphate donor in a regioselective way.[13] In order to investigate the possible enantioselectivity of these enzymes in phosphorylation reactions, 1-(4-nitrophenoxy)-2,3-propanediol was synthesized as a glycerol derivative (Chart 2) and incubated with PhoN-Sf and PhoN-Se. The rather large \( p \)-nitrophenyl group may cause discrimination between the (R)- and (S)-enantiomer in the phosphorylation of the primary hydroxyl group of the diol. Both enzymes phosphorylated the substrate and maximal concentrations reached after 90 min were 10.7 mM for PhoN-Sf and 16.5 mM for PhoN-Se, respectively, from 100 mM substrate and 100 mM PPi. Addition of two more portions of PPi (100 mM) after 2 and 4 h, respectively, increased the yield further to 23.6 mM for the PhoN-Sf reaction. Unfortunately, enantioselectivity was not observed.

1-Phenylethanol was chosen to investigate the possible enantioselectivity in the phosphorylation of a secondary alcohol (Chart 2). As already demonstrated, the rate of phosphorylation of a secondary alcohol group is much slower than that of a primary alcohol.[13] Maximal concentrations of phosphorylated product of 1.5 mM and 8 mM with PhoN-Sf and PhoN-Se, respectively, were observed between 1.5 and 2.5 h. In order to obtain a higher conversion a reaction was carried out in which three times 100 mM PPi was added and this increased the yield to 27 mM using PhoN-Se. No change occurred in the 1:1
ratio of the two enantiomers present during the reaction as analyzed by chiral HPLC, which means that the enzyme does not preferentially phosphorylate one of the two enantiomers.

Chart 2. Substrates used to investigate the enantioselectivity of the phosphorylation by PhoN-Sf and PhoN-Se using PP, as a phosphate donor.

Selectivity in dephosphorylation of geometric isomers
The possible preference of the enzymes in the dephosphorylation of geometric isomers was also investigated and 2-methylcyclohexanemethanol phosphate was chosen as substrate (Chart 3). The time dependent dephosphorylation of 2-methylcyclohexanemethanol phosphate was studied by HPLC. Both enzymes showed two phases in the dephosphorylation rate, a fast phase and a much smaller but slower phase. When the phases were compared with the initial composition of the mixture (cis:trans; 89.9:10.1) they coincide with the initial composition, indicating a slower dephosphorylation of the trans-isomer. However, this is not conclusive, since the dephosphorylation rate could also have slowed down due to a large $K_d$-value of the substrate. When this dephosphorylation was checked by $^1$H NMR, the cis:trans ratio of dephosphorylated product changed from 92.1:7.9 and 92.6:7.4 for PhoN-Sf and PhoN-Se respectively after 1 h of reaction (50 % conversion) to 89.9:10.1 after 24 h of reaction (100% conversion). This observation also indicates that the trans-isomer is the isomer that is more slowly dephosphorylated. Dephosphorylation of a mixture with an initial ratio of 1:1 should give more conclusive evidence for enantioselectivity.

Chart 3. Structures of the geometric isomers of 2-methylcyclohexanemethanol phosphate (R = CH$_2$OPO$_3$H$_2$) and 2-methylcyclohexanol phosphate (R = OPO$_3$H$_2$).

To make the structure of the substrate more rigid, and thereby possibly increasing the discrimination between the isomers, both trans- and cis-2-methylcyclohexanol phosphate were prepared and their dephosphorylation reaction was studied in a mixture consisting of the phosphate esters in a trans:cis ratio of 2:1. During dephosphorylation by PhoN-Se, the ratio of the phosphate esters did not change and a 2:1 ratio (trans:cis) of alcohol product was observed (Figure 3). The conversion was 91% after 5 h. However, PhoN-Sf showed a difference in dephosphorylation rate in favour of the cis-isomer (Figure 3). For every trans-phosphate ester, two cis-esters were hydrolyzed resulting in a trans:cis ratio of the alcohol product of 0.5. The trans:cis ratio of the remaining phosphate ester increased in favour of the trans-isomer, which is the more slowly hydrolyzed isomer. The conversion reached 38% after 6 h. The dephosphorylation of 2-methylcyclohexanolphosphate by potato acid phosphatase has been described by Klibanov et. al.[23] It is interesting to note that this enzyme showed opposite selectivity compared to PhoN-Sf. The potato acid phosphatase completely hydrolyzed the trans-isomer of the 2-methylcyclohexanol phosphate ester in 23 h, whereas its cis-counterpart did not react with the enzyme at all under the same
conditions. This dramatic difference in reactivity of the geometric isomers was explained by saying that the methylgroup in the cis-compound precludes the phosphatase from attacking the ester bond, whereas in the trans-compound this effect clearly does not take place. Dephosphorylation of the geometric isomers of 3- and 4-methylcyclohexanol phosphate esters by potato acid phosphatase did not show this difference. Similarly, the acid phosphatase from wheat germ and the alkaline phosphatases from E. coli and calf intestine had a preference for the hydrolysis of trans-2-methylcyclohexanol phosphate. So the PhoN-Sf preference for the cis-isomer is surprising. Although the resolution is not optimal, the reaction can be further optimized by e.g. directed evolution of the PhoN-Sf enzyme. It is interesting to note that when an esterase is used in a reaction with rac-2-methylcyclohexanol phosphate also the trans-isomer is preferred, but separation of the geometric isomers has not been achieved. In contrast, esterases can be used to resolve the (1R)-acetate and the (1S)-alcohol of both the cis- and trans-isomers in high optical purity.

![Figure 3](image-url)

**Figure 3.** Trans/cis ratios during dephosphorylation of a mixture of methylcyclohexanol phosphate isomers by PhoN-Sf and PhoN-Se. Reaction mixture contained 100 mM of methylcyclohexanol phosphate originally present in a trans/cis ratio of 2:1 in 100 mM acetate buffer (pH 6.0) at 30°C. The reaction was started by the addition of either 1 μM PhoN-Sf (open symbols) or PhoN-Se (closed symbols). The trans/cis ratios of the resulting alcohols (squares) and remaining phosphate esters (circles) were determined by HPLC.

**Conclusions**

The use of the acid phosphatase from *Salmonella enterica* ser. *typhimurium* LT2 in enantioselective hydrolysis of O-phospho-DL-threonine shows that this enzyme can be used for the preparative resolution of DL-threonine. Up to 30% conversion of O-phospho-DL-threonine (which is 60% based on the L-enantiomer) the ee value is 100%. The preference for hydrolysis of O-phospho-L-threonine is mostly caused by the configuration at the β-carbon since it is shown that the enantioselectivity for hydrolysis of O-phospho-DL-serine is much less and reversed; O-phospho-D-serine is preferred. The hydrolysis of O-phospho-DL-serine by PhoN-Se is not suitable for preparative resolution. Directed evolution of the PhoN-Se enzyme has been performed to optimize this resolution reaction and is described in chapter 6.

Other enantioselective hydrolysis reactions are not found up to now, but the acid phosphatase from *Shigella flexneri* recognizes the geometric isomers of methylcyclohexanol phosphate and is two times more reactive towards the cis-isomer.
Acknowledgements

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Experimental section

**General Information.** Expression and purification of recombinant *Shigella flexneri* PhoN (PhoSf) [14] and *Salmonella enterica* PhoN (PhoN-Se) [17] were as described. All chemicals were purchased from commercial suppliers and used without purification.

**HPLC method 1:** The amount of free phosphate was determined by HPLC using an Alltech OA 1000 organic acid column (0.65 cm x 30 cm) equipped with a Dionex 580 LPG pump and Dionex UVD-340/Shodex RI-101 detector. The column was eluted with 20 mM H2SO4 at a flow rate of 0.4 mL min⁻¹. The HPLC effluent was monitored at 210, 215, 275 and 320 nm. The Chromeleon software program (Dionex) was used for HPLC data acquisition and evaluation. Samples were diluted 50 times in H2O before injection.

**HPLC method 2:** The amounts of D- and L- amino acids were determined by chiral HPLC using a Daicel crownpak CR column (0.4 cm x 15 cm) at -5°C, equipped with a Dionex 580 LPG pump and Dionex UVD-340/Shodex RI-101 detector. The column was eluted with 100 mM HClO₄ at a flow rate of 0.5 mL min⁻¹. The HPLC effluent was monitored at 340 nm. The Chromeleon software program (Dionex) was used for HPLC data acquisition and evaluation. Samples were diluted 50 times in H2O before injection.

**HPLC method 3:** The amounts (R)- and (S)-1-(4-nitrophenoxy)-2,3-propanediol were determined by chiral HPLC using a Chiralcel OB column (0.46 cm x 25 cm) equipped with a Pharmacia LKB HPLC Pump 2248 and a LKB Bromma optical unit 2140 Rapid Spectral Detector. The column was eluted with hexane:IPA 9:1 at a flow rate of 1 mL min⁻¹. The HPLC effluent was monitored at 220 nm. The Borwin software program was used for HPLC data acquisition and evaluation. The retention times for the (S)- and (R)-enantiomers were 26.7 and 29.8 min respectively.

**HPLC method 4:** The amounts (R)- and (S)-1-phenylethanol were determined by chiral HPLC using a Chiralcel OD column (0.46 cm x 25 cm) equipped with a Pharmacia LKB HPLC Pump 2248 and a LKB Bromma optical unit 2140 Rapid Spectral Detector. The column was eluted with hexane:IPA 24:1 at a flow rate of 1 mL min⁻¹. The HPLC effluent was monitored at 220 nm. The Borwin software program was used for HPLC data acquisition and evaluation. The retention times for the (R)- and (S)-enantiomers were 9.9 and 11.9 min respectively.

Nuclear magnetic resonance spectra (¹H NMR and ¹³C NMR) were determined in the indicated solvent using a Varian Unity Inova 500 spectrometer.

**Chemical synthesis**

\[
\text{Rac-,(R)- and (S)-1-(4-nitrophenoxy)-2,3-propanediol,}^{[20]} \text{ p-Nitrophenol (0.939 g, 6.75 mmol) and rac-glycidol (0.50 g, 6.75 mmol) were dissolved in anhydrous ethanol (5 mL) and triethylamine (0.087 mL, 0.63 mmol) was added. The reaction mixture was reflushed for 4 hours. The reaction was monitored by TLC using CH₂Cl₂ : MeOH 19 : 1 as eluens. Purification was performed by silica column chromatography using the same eluens. Yield: 0.18 g, 12.5%. ¹H NMR (500 MHz, d₆ DMSO): δ 3.82 (m, 1H), 4.02 (q, J = 6.3 and 10.0, 1H), 4.16 (q, J = 3.8 and 10.1, 1H). 4.74 (t, J = 5.6, 1H), 5.06 (d, J = 5.1, 1H), 7.15 (m, 2H, ar), 8.2 (m, 2H, ar). The (R)- and (S)- compounds were synthesized in a similar way, only the rac-glycidol was replaced with enantiopure glycidol and double amounts of reagents were used. Yield (R): 0.51 g, 17.7%; (S): 0.34 g, 11.8%.
\]

\[
\text{Rac-2-methylcyclohexanemethanol,}^{[27]} \text{ To dry THF (20 mL), LiAlH₄ (0.4 g) was added under a nitrogen atmosphere. 2-Methyl-1-cyclohexane carboxylic acid (1.03 g, 7.2 mmol; mixture of cis and trans) was dissolved in dry diethyl ether (25 mL) and added dropwise. The suspension was stirred for 1 hour at RT after which the mixture was heated to 50°C for 30 minutes. Diethyl ether (3 x 100 mL) was used for extraction. The combined organic layer was washed with a NaHCO₃-solution and was dried over anhydrous MgSO₄, which was removed by filtration. The organic solvent was removed in vacuo to provide 2-methylcyclohexanemethanol. Yield: 0.836 g, 90%. ¹H NMR (500 MHz, CDCl₃): δ 0.88 (d, J = 7.3, 3H, cis CH₃), 0.88 (d, J = 7.2, 3H, trans CH₃).}
\]
0.94 (d, J = 6.3, 3H, \textit{trans} CH$_3$), 1.10 (m, 1H), 1.35 (m, 2H), 1.48 (m, 2H), 1.85 (m, 1H), 1.88 (m, 2H), 3.52 (m, 2H, \textit{trans} CH$_3$), 3.78 (m, 2H, \textit{cis} CH$_2$). Ratio of \textit{cis}/\textit{trans} 89.9:10.1.

\textbf{Rac-2-methylcyclohexanemethanol phosphate, sodium salt.}\textsuperscript{[28]} 2-Methylcyclohexanemethanol (0.5 g, 3.9 mmol) was dissolved in THF (26.5 mL) and DIPEA (diisopropylethylamine, 5.3 mL, 30 mmol) was added under permanent stirring. The solution was cooled to -10°C and POCl$_3$ (2.65 mL, 30 mmol) was added dropwise in 5 minutes. The mixture was kept at RT for 5 minutes, after which water (40 mL) was added dropwise (cooling with liquid nitrogen). The solution was adjusted to pH 5.0 by gradual addition of 3M KHCO$_3$ (28 mL) at RT. After evaporation of the solvent THF, the mixture was extracted twice with CHCl$_3$ (30 mL). The combined organic layers were dried over MgSO$_4$, and concentrated in vacuo. The residue was dissolved in water (10 mL), brought to pH 7.0 with NaOH and was freeze-dried in order to store the phosphate ester as a sodium salt. Yield: 0.81 g, 50%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 0.70 (d, J = 7.1, 3H, \textit{cis} CH$_3$), 0.73 (d, J = 6.5, 3H \textit{trans} CH$_3$), 1.19 (m, 3H) 1.45 (m, 2H), 1.71 (m, 3H), 3.68 (m, 2H, \textit{cis} CH$_2$), 3.82 (m, 2H, \textit{trans} CH$_2$).

\textbf{Trans-2-methylcyclohexanol phosphate, barium salt.}\textsuperscript{[29]} Trans-2-methylcyclohexanol (1 g, 8.757 mmol) was added dropwise to POCl$_3$ (1.47 g, 9.587 mmol) and was allowed to stand for 1 hour at ambient temperature. The reaction mixture was added to H$_2$O (90 mL) in which Ba(OH)$_2$ (9.07 g, 28.75 mmol) was pulverized. Dry ice was added to precipitate the excess Ba$^{2+}$. The adduct of an equal volume of ethanol caused the phosphate ester of trans-2-methylcyclohexanol to precipitate as a white solid, which was collected by filtration and washed with ethanol. Yield: 0.88 g, 34%. $^1$H NMR (400 MHz, D$_2$O): $\delta$ 0.88 (d, J = 6.5, 3H), 0.96 (m, 1H), 1.17 (m, 4H), 1.49 (m, 1H), 1.63 (m, 2H), 2.06 (m, 1H), 3.46 (m, 1H).

\textbf{Cis-2-methylcyclohexanol phosphate, barium salt.}\textsuperscript{[29]} Cis-2-methylcyclohexanol phosphate ester was prepared as described above. Yield: 0.39 g, 15%. $^1$H NMR (400 MHz, D$_2$O): $\delta$ 0.84 (d, J = 7.0, 3H), 1.23 (m, 2H), 1.39 (m, 4H), 1.53 (m, 1H), 1.65 (m, 1H), 1.81 (m, 1H), 4.00 (m, 1H).

**Enzymatic reactions**

**Dephosphorylation of O-phospho-DL-threonine and O-phospho-DL-serine.** A mixture containing 100 mM O-phospho-DL-threonine or O-phospho-DL-serine in 100 mM acetate buffer pH 6.0 at 30°C was completed with either 1 mM PhoN-Sf or PhoN-Se. pH Dependent dephosphorylation was carried out at pH 3.3, 3.9, 4.3, 5.0 and 6.25 and 5 mM PhoN-Sf or PhoN-Se was used. Reaction rates were determined by time course HPLC measurements (method 1) of the released phosphate. Chiral HPLC (method 2) was used to determine the ee values of the released amino acids.

**Phosphorylation of rac-1-(4-nitrophenoxy)-2,3-propanediol.** Phosphorylation of a mixture containing 100 mM rac-1-(4-nitrophenoxy)-2,3-propanediol, 100 mM PP$_i$ in 100 mM acetate buffer (pH 5.3) at 30°C was started by the addition of 1 mM PhoN-Sf or PhoN-Se. Conversion of the reaction was checked by time course HPLC (method 1). Enantioselectivity of the reaction was measured with chiral HPLC (method 3). 20 mL of sample was extracted with 200 mL CH$_2$Cl$_2$, from which 200 mL were evaporated and the residue was solubilized in CDCl$_3$.

**Phosphorylation of rac-1-phenylethanol.** Phosphorylation of a mixture containing 100 mM rac-1-phenylethanol, 100 mM PP$_i$ in 100 mM acetate buffer (pH 5.3) at 30°C was started by the addition of 1 mM PhoN-Sf or PhoN-Se. Conversion of the reaction was checked by time course HPLC (method 1). A higher yield in phosphonomoester was achieved by addition of two extra portions of PP$_i$, after 2 and 4 hours of reaction, respectively. After 6 h the conversion and ee were determined.

**Phosphorylation of rac-2-methylcyclohexanemethanol phosphate.** Dephosphorylation of 200 mM rac-2-methylcyclohexanemethanol phosphate in 100 mM acetate buffer (pH 6.0) at 30°C was started by the addition of 1 mM PhoN-Sf or PhoN-Se. Conversion and trans/cis ratio of the reaction were checked by time course HPLC (method 1). Samples for $^1$H NMR were prepared by extracting 0.5 mL of the reaction mixture after 1 h with 1 mL CH$_2$Cl$_2$. This extract (0.5 mL) was evaporated and the residue was solubilized in CDCl$_3$.

**Docking.** The X-ray structure of PhoN from \textit{Salmonella enterica ser. typhimurium} MD6001\textsuperscript{[21]} (PDB: 2A96) and \textit{Escherichia coli} [25] (PDB: 1EOI) were used to prepare the models of \textit{Salmonella enterica ser. typhimurium Chapter 5}
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LT2[16] using the WhatIf program.[30] The model was docked with the phosphate esters of the amino acids using the program FlexX.[31] Structures of the phosphate esters were generated with the program Molden.[32]

References