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Chemo-enzymatic approaches for the creation of novel chiral building blocks and reagents for pharmaceutical applications

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Abstract

This paper describes three recent examples of innovative biocatalyst applications and technology developments from our laboratory giving access to useful chiral building blocks (examples 1 and 2) and chiral reagents (example 3).

1. Preparative to larger scale synthesis of enantiomerically pure chiral arylalkylamines using whole cell biotransformations with microorganisms containing novel enantioselective amidohydrolases.
2. Preparative to larger scale chemo-enzymatic synthesis of D- and L-*tert*-leucines using the very cheap commercial bulk enzyme Alcalase.
3. Preparative scale chemo-enzymatic synthesis of rare and expensive inositol phosphates, including 5′-TAMRA-labeled (P1-tethered) D-PIP_3, using commercial lipase preparations.

Keywords: Chiral amines; D- and L-*tert*-leucine; Inositol phosphates; 5′-TAMRA-labeled (P1-tethered) D-PIP_3; Enantioselective amidohydrolases; Alcalase; Lipases

1. Introduction

Chiral reagents and chiral building blocks play important roles in the drug discovery process and in the manufacture of modern pharmaceutical drugs. Due to the improved and increased application of modern target-structure-based biorational techniques throughout the drug discovery process, it can easily be predicted that the role of chirality will become even more important for the drugs of the future. Therefore, new efficient and selective ways are needed to produce interesting reagents and chiral building blocks. Very attractive possibilities are offered by the use of biocatalytic reactions for some of the key synthesis steps requiring a very high degree of chemical and/or structural selectivity.

Chiral amines represent a highly versatile and attractive group of chiral building blocks for drug R&D. Some of the companies dealing with specialty chemicals offer a limited selection of enantiomerically pure chiral amines. However, these compounds are often available in small quantities only and at prohibitive prices. Chiral amines are of interest, e.g. as building blocks for $GABA_B$ antagonists or substance P antagonists (Fig. 1). They are also of interest as versatile

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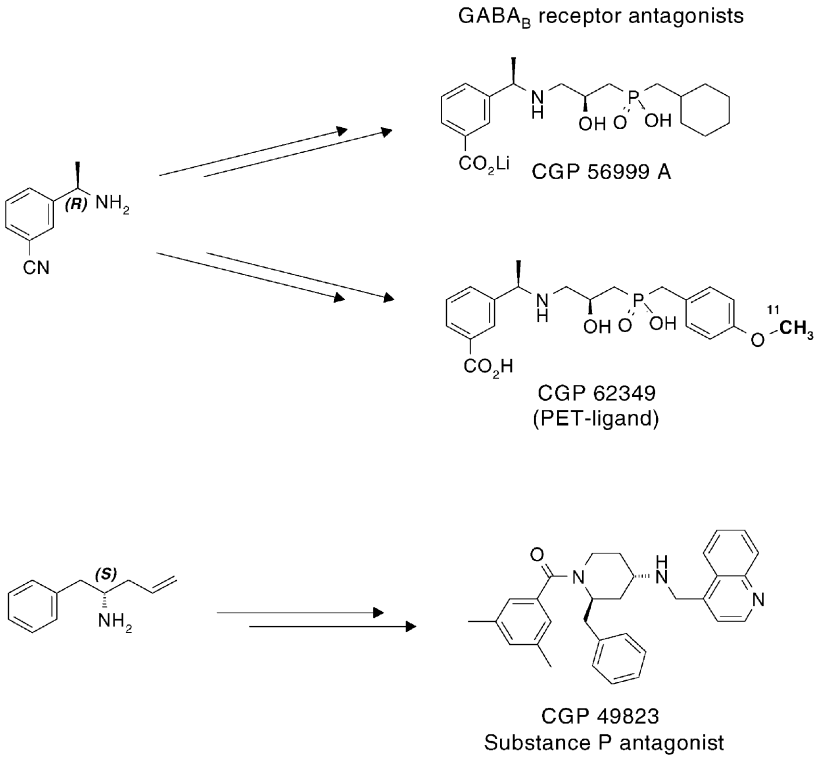

Fig. 1. Chiral amines as building blocks for $GABA_B$ receptor antagonists and substance P antagonist.

new building blocks for combinatorial chemistry and help to significantly increase the chemical diversity of the generated compound libraries. With a newly established enantioselective microbial bioconversion technology involving 3 novel amidohydrolases, 14 different types of chiral amines have been prepared in 50–100 g scale with high to very high enantiomerical purity and very high conversion yields.

D-*tert*-Leucine is an attractive starting material for the creation of chiral auxiliaries and as a building block for combichem projects. On the market, it is currently not available in significant amounts. Therefore, a new chemo-enzymatic approach using a commercial protease for the enantioselective cleavage of racemic *N*-acetyl-*tert*-leucine esters was established. The enzymatic reaction was scaled up to the kg level.

Chemo-enzymatic routes are also of great interest for the preparation of rare and exotic research biochemicals. This was demonstrated in our laboratory, e.g. for the preparation of inositol polyphosphates, where lipases proved to be very valuable and versatile enantioselective esterification biocatalysts. So far, a set of 13 different types of inositol phosphates was made available in preparative scale, including 5′-TAMRA-labeled (P1-tethered) D-PIP_3.

2. Biotransformations with microorganisms containing novel amido-hydrolases for the preparation of enantiomerically pure arylalkylamines

2.1. Biocatalytic approaches described in literature

Enantiomerically pure chiral amines are of high interest for both the pharmaceutical and the agrichemical industries (for a recent review see [1]). Besides classical approaches using separation of enantiomers,

several biocatalytic ways for the production of such compounds have been proposed and developed more or less successfully. Some of the most relevant examples are as follows.

- The production of D-2-aminobutanol via microbial hydrolysis of the corresponding *N*-acyl derivatives [2].
- The enantiomeric separation of amines from racemates having the amino group on a secondary carbon atom through a process involving the action of ω-amino acid transaminases [3].
- The biological syntheses of (*S*)-1-phenylethylamine from L-alanine and acetophenone by *Acinetobacter* sp. MBA-15 or from alkyl-(1-phenylethyl)carbamate in the presence of bacteria of the genera *Rhodococcus* or *Arthrobacter*, as described in literature [4,5], are unfortunately characterized by low yields and long incubation times.
- The production of optically active 1-aryl-2-aminopropane by enantioselective transfer of the amino group in the racemate by means of the amino acid transaminase from *Bacillus megaterium* [6].
- The preparation of chiral amines from racemates by specific amidation of the (*R*)-enantiomer via acyl transfer reactions using lipase B from *Candida antarctica* [7–10], and by (*S*)-specific amidation catalyzed by subtilisin [11,12].
- The production of optically active primary and secondary amines by enantioselective acylation of a racemic amine with an activated ester as an acyl donor in the presence of the lipase from *Pseudomonas* sp. [13]. For the multi-tonne scale production of (*S*)- and (*R*)-1-phenylethylamine and other chiral amines from racemate via the enantioselective acylation of the (*R*)-form, BASF uses the lipase from *Pseudomonas* sp. and methoxyacetic acid ethylester as the acylating agent [14].
- The enzymatic asymmetric synthesis of α-methyl arylalkylamines and α-methyl arylalkylalcohols by arylalkyl acylamidases. The (*S*)-enantiomers of 1-methylbenzylamine, 1-methyl-3-phenylpropylamine and 1-methyl-3-phenylpropanol were synthesized in high enantiomeric purity with *Nocardia erythropolis* IAM 1440 or *Cellulomonas fimi* AKU 671 (and various other microbes). (*R*)-1-Methyl-3-phenylpropylamine was prepared with crude amidase from *Pseudomonas putida* Sc2 AKU 881 [15–17].

2.2. Chiral amines as pharmaceutical building blocks: target compounds

In our study, the major target compounds in demand for pharmaceutical applications and drug profiling were (*R*)- and (*S*)-1-(3-cyanophenyl)ethylamine, (*R*)- and (*S*)-1-(4-cyano-phenyl)ethylamine, (*S*)-2-amino-1-phenyl-4-pentene, and (*S*)-2-amino-1-(4-chlorophenyl)-4-pentene.

(*R*)-1-(3-Cyanophenyl)ethylamine (Fig. 1) was required as a building block for the $GABA_B$ receptor antagonists CGP 56999A (for the treatment of Alzheimer patients), CGP 62349 (PET-ligand), and related structures [18,19], as well as for radioligands [19]. The (*S*)-enantiomer and the two enantiomers of 1-(4-cyanophenyl)ethylamine were needed for comparative studies.

(*S*)-2-Amino-1-phenyl-4-pentene (Fig. 1) was required as a building block in an alternative synthesis of the substance P antagonist CGP 49823 (anxiolytic, antidepressant), and related structures [20,21]. (*S*)-2-Amino-1-(4-chlorophenyl)-4-pentene was needed for comparative studies.

2.3. Three novel amidohydrolases: physico-chemical properties and selectivities

In the course of an extended screening program in our laboratory, three novel highly enantioselective microbial amidohydrolases were discovered [22–24]. The production of these enzymes from *Rhodococcus equi* (deposited as DSM 10278), *Arthrobacter aurescens* (deposited as DSM 10280), and *Rhodococcus globerulus* (deposited as DSM 10337) was optimized and the isolated enzymes were then broadly evaluated for synthetic applications [25]. The three enzymes (showing different and opposite selectivities) proved to be highly suitable for the preparative scale synthesis of a broad variety of enantiomerically pure chiral amines from the corresponding racemic *N*-acetyl derivatives. The operational stability of all three enzymes is very good which makes them highly suitable for application as biocatalysts in industrial scale.

The inducible (*S*)-specific *N*-acetyl-1-phenylethylamine amidohydrolase from *R. equi* Ac6 [22] deacetylates the (*S*)-enantiomers of a broad range of *N*-acetylamines with aromatic side-chains. With this

new enzyme, the pure (*S*)-enantiomers of 1-(3-cyanophenyl)ethylamine and 1-(4-cyanophenyl)ethylamine were easily synthesized on preparative scale. The enantioselectivity (*E*) is 350 for (*S*)-*N*-acetyl-1-phenylethylamine and >500 for (*S*)-*N*-acetyl-1-(3-cyanophenyl)ethylamine and for (*S*)-*N*-acetyl-1-(4-cyanophenyl)ethylamine.

Unfortunately, (*R*)-1-(3-cyanophenyl)ethylamine and (*R*)-1-(4-cyanophenyl)ethylamine can not be prepared by acidic or alkaline hydrolysis of the corresponding (*R*)-*N*-acetylamides (the left over enantiomer from the racemate under catalysis of the acylase from *R. equi* Ac6) because this treatment results in the degradation of the cyano group. Therefore, an (*R*)-specific enzyme was needed and found in our screening program.

The constitutive (*R*)-specific *N*-acetyl-1-phenylethylamine amidohydrolase from *A. aurescens* AcR5b [23] has a substrate range comparable to the one of the *R. equi* Ac6 enzyme but the enantioselectivity is in the opposite direction. With this enzyme also the pure (*R*)-enantiomers of 1-(3-cyanophenyl)ethylamine and 1-(4-cyanophenyl)ethylamine could be synthesized on preparative scale. The enantioselectivity (*E*) is >500 for (*R*)-*N*-acetyl-1-phenylethylamine and 320 for (*R*)-*N*-acetyl-1-(4-cyanophenyl)ethylamine.

Since the amidohydrolases from *R. equi* Ac6 and *A. aurescens* AcR5 do not hydrolyze racemic *N*-acetyl-2-amino-1-phenyl-4-pentene enantioselectively, a third type of amidohydrolase was demanded and also found in our screening program.

The inducible (*S*)-specific *N*-acetyl-2-amino-1-phenyl-4-pentene amidohydrolase from *R. globerulus* K1/1 [24]—similar to the other two amidohydolases—deacetylates the (*S*)-enantiomers of a broad range of *N*-acetylamines with aromatic side-chains. However, the substrate range of this amidohydrolase is clearly different from that of the *R. equi* Ac6 enzyme. With this third enzyme it became very easy to prepare larger amounts of the desired (*S*)-2-amino-1-phenyl-4-pentene, or (*S*)-2-amino-1-(4-chlorophenyl)-4-pentene, as well as structurally related compounds. The enantioselectivity (*E*) is >500 for (*S*)-*N*-acetyl-1-phenylethylamine and >500 for (*S*)-*N*-acetyl-2-amino-1-(4-chlorophenyl)-4-pentene.

None of the three enzymes accepts *N*-methyl-*N*-acetylamines or *N*-acetylated amino acids as substrates.

In all three cases, the enantioselective hydrolysis of the racemic substrate can either be performed with the isolated amidohydrolases or—more conveniently—with whole (resting) cells of the amidohydrolase containing microorganisms. In the whole cell biotransformations, 10% methanol can be added for solubilization of poorly soluble substrates.

2.4. Preparative scale syntheses of chiral amines using the three novel amidohydrolases

Example: Preparation of (*S*)-2-amino-1-(4-chlorophenyl)-4-pentene in a laboratory pilot scale.

R. globerulus K1/1 was cultivated in a 20 l fermenter with 1 g/l *N*-acetyl-phenylethylamine as inducer [24] and 70 g wet cell mass with 6000 U amidohydrolase were obtained. Eighty grams of racemic *N*-acetyl-2-amino-1-(4-chlorophenyl)-4-pentene were dissolved in 2 l methanol and added under stirring to 20 l phosphate buffer, pH 7.0. Then 60 g of wet cells of *R. globerulus* K1/1 were added and the mixture was stirred at 800 rpm and 30 °C. During the enzymatic hydrolysis of the remaining (*R*)-amide and the (*S*)-amine produced (derivatized as acetamide) were analyzed by chiral HPLC (Chiralcel OB, hexane:2-propanol (95:5), flow 1 ml/min). After 24 h (50% conversion) the optical purity of the remaining substrate and of the amine produced was >99%. The pH was adjusted to about 12 and the mixture was extracted five times with dichloromethane. The organic solutions were concentrated to 2 l and extracted with 2 M HCl to remove the amine. The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure yielding 39 g (*R*)-*N*-acetyl-2-amino-1-(4-chlorophenyl)-4-pentene (99% e.e.). The HCl extracts were neutralized and extracted with dichloromethane. After usual work-up and distillation 31.7 g (*S*)-2-amino-1-(4-chlorophenyl)-4-pentene were obtained (>99.9% e.e.).

A small library of chiral amines was created using whole cell biotransformations in analogy to the example given above. Fig. 2 shows the main parameters of five such biotransformations. Fig. 3 presents all the chiral amines generated so far on preparative scale as building blocks for medicinal and combinatorial chemistry. The racemic amides (enzyme substrates) were prepared by acetylation of the corresponding amines. The racemic amines were easily synthesized by catalytic reduction of the oximes which are

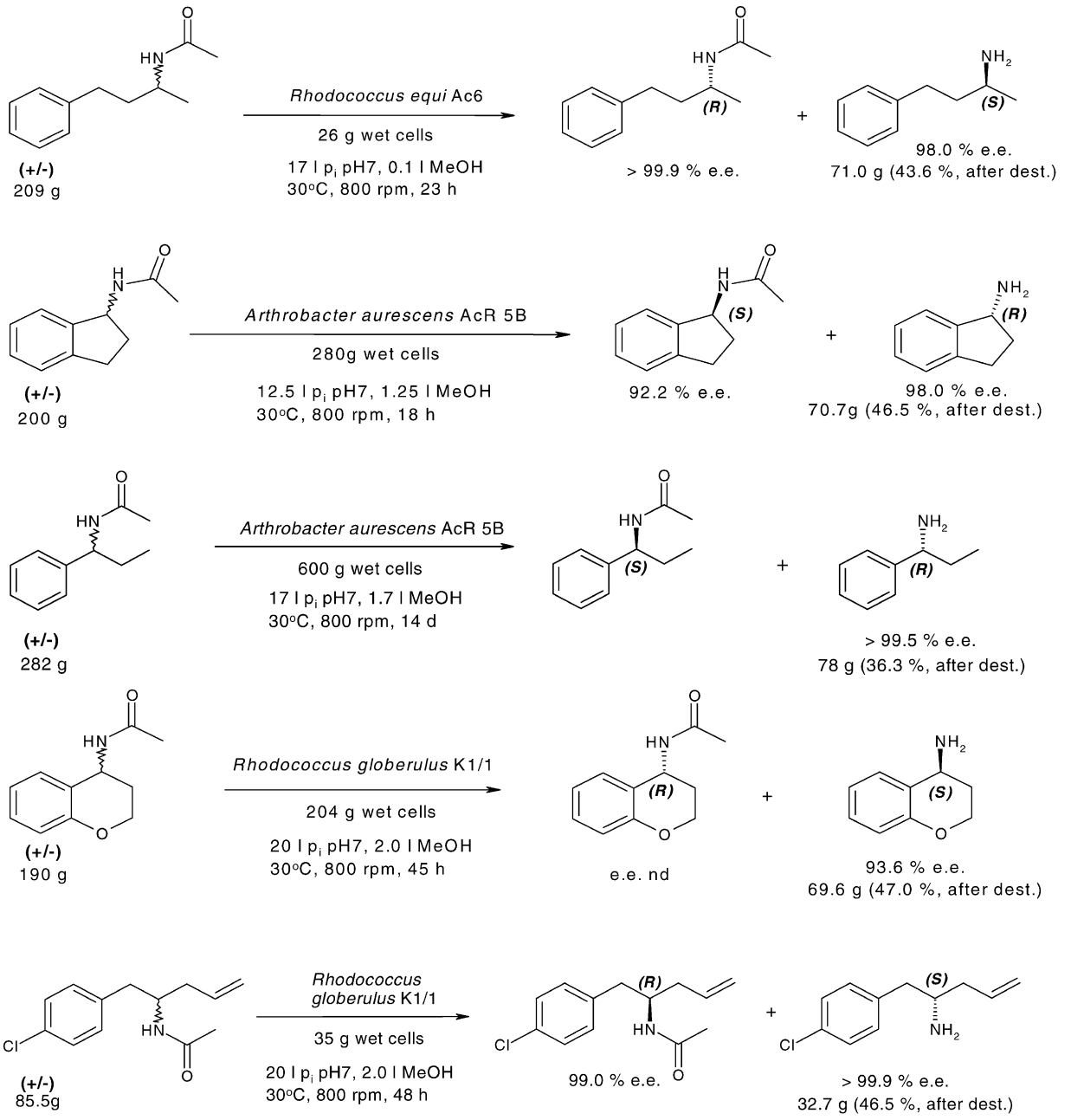

Fig. 2. Typical biotransformations with enantioselective amidohydrolases in whole cells of *R. equi*, *A. aurescens* and *R. globerulus*.

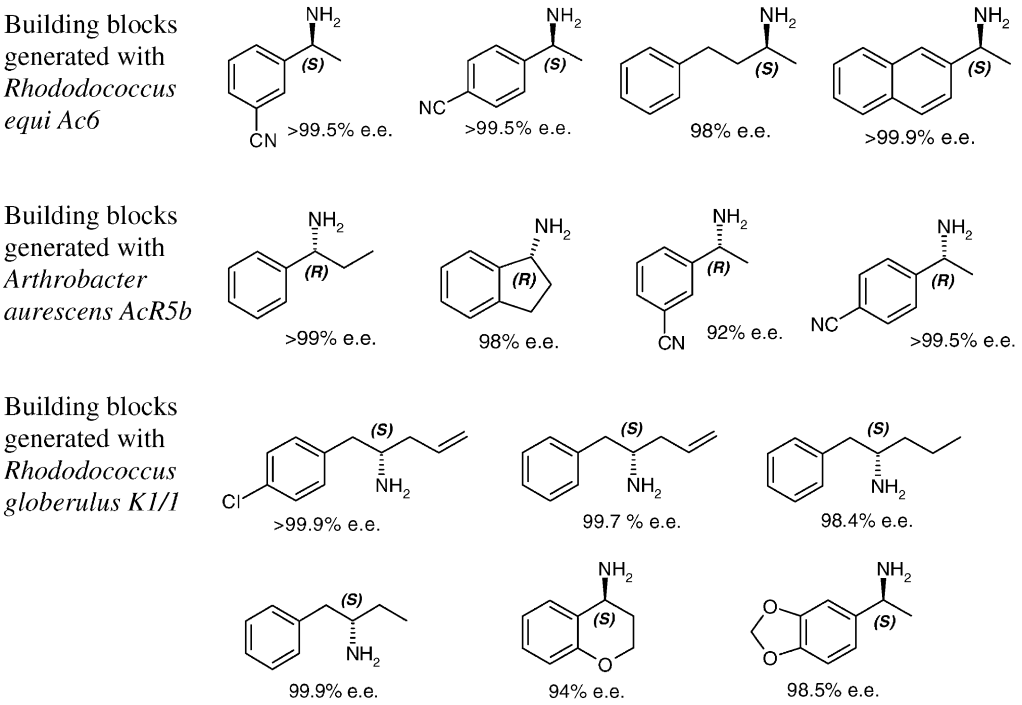

Fig. 3. Chiral building blocks generated in preparative scale (50–100 g) using microbial amidohydrolases (whole cell biotransformations).

obtainable from the commercially available corresponding ketones.

3. Chemo-enzymatic synthesis of D- and L-*tert*-leucines using the very cheap commercial bulk enzyme Alcalase

3.1. Tertiary leucines as pharmaceutical building blocks

Optically active *tert*-leucine and derivatives thereof are attractive starting materials for the creation of chiral auxiliaries, for the synthesis of ligands in asymmetric catalysis, and as building blocks for drugs, e.g. acting as protease inhibitors in various indications [26]. Due to the increasing interest in this particular building block, several preparation procedures via chemical resolution [27–32], by chiral induction [33–37] and by enzymatic approaches [38–42] have been reported. L-*tert*-Leucine is now produced on an industrial scale (Degussa) by cofactor-dependent reductive amination of the corresponding α-keto acid catalyzed by leucine dehydrogenase [41,43]. In contrast, D-*tert*-leucine is not available by a similar process [26,44] and there is still no large scale method commercially established for its preparation [45].

3.2. Preparative scale syntheses of D- and L-*tert*-leucines—a novel chemo-enzymatic approach

Our first approach, the selective enzymatic cleavage of racemic *N*-acetyl-leucine was unsuccessful. A large number of isolates from habitat samples showed activity with *N*-acetyl valine but not with *N*-acetyl-*tert*-leucine. A second approach [46] using proteases for the enantioselective cleavage of racemic *N*-acetyl-*tert*-leucine esters was evaluated (Fig. 4a). Out of 45 tested commercial enzymes, 4 [46] showed hydrolytic activity toward the racemic methylester: Bioprase SP4 (Nagase, 13% relative activity), Prozyme 6 (Amano, 15% relative activity), Protease N (Amano, 60% relative activity), and Alcalase (Novo, 100% relative activity). The most active enzyme was the

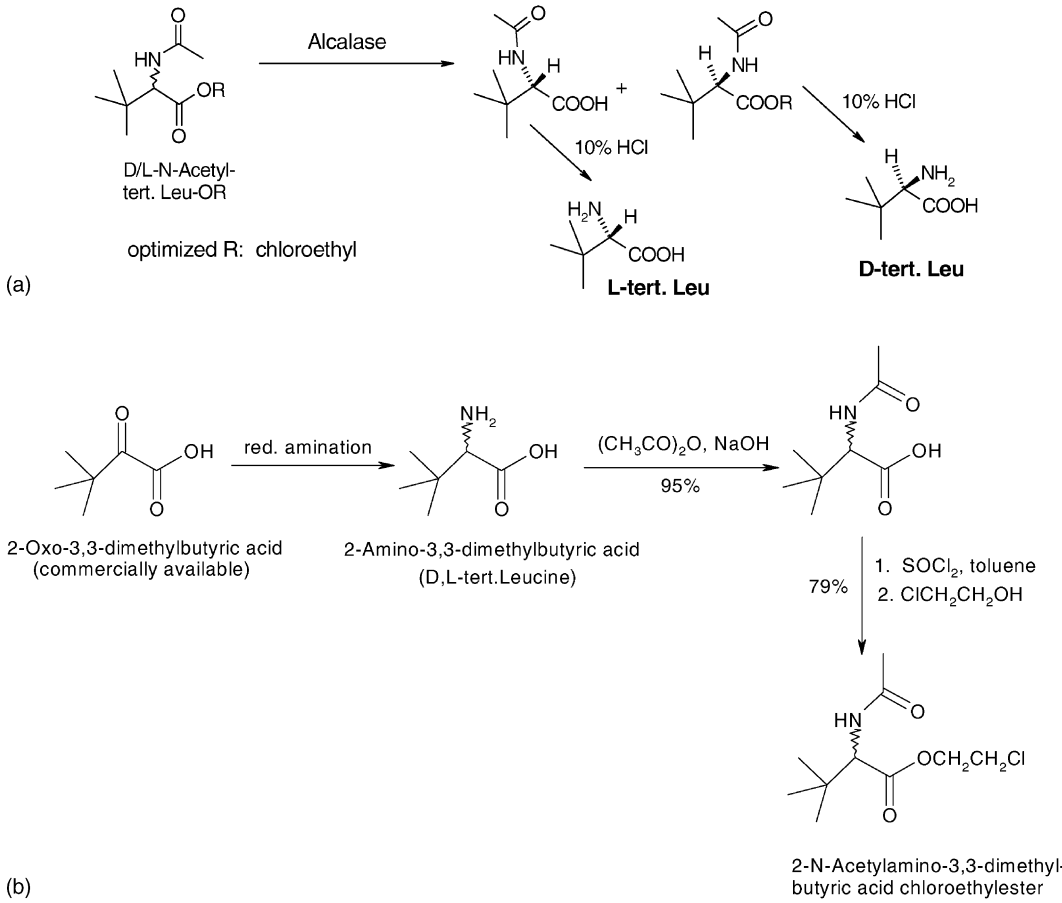

Fig. 4. (a) Preparation of enantiomerically pure *tert*-leucines from racemic *N*-acetyl-*tert*-leucine esters [46]. (b) Synthesis of racemic *N*-acetyl-*tert*-leucine chloroethylester [46].

protease from *Bacillus licheniformis* (Alcalase). However, for a large scale preparation the activity with the methylester was still too low. A comparison of the hydrolytic activity with various esters, i.e. ethyl-, octyl-, trichloroethyl- and chloroethylester, at three different pH values [46] showed a six-fold increase in activity for the chloroethylester at pH 8 compared to the methylester at pH 7. The pH values >8 are not recommended in order to avoid chemical hydrolysis of the substrate. The observation that the rate of hydrolysis could be increased by incorporating a chlorine seems to be similar, albeit with a reduced effect, to that described earlier for the lipase-catalyzed hydrolysis of chloro acetates versus acetates [47].

In a preparative experiment [46], 200 g of racemic *N*-acetyl-*tert*-leucine chloroethylester was dissolved in toluene (300 ml), a phosphate buffer at pH 8.0 (800 ml) and 500 ml of Alcalase (Novo Nordisk, type DX, PMN04666, 2.67 a.u./g proteolytic activity, pH adjusted) were added, and the mixture was stirred at 35 °C. The pH value was kept constant with a pH-stat by adding a 1 M NaOH solution. After about 50 h, the conversion reached 51.3%. Extraction with dichloromethane and a standard work-up[1] gave optically pure D-*N*-acetyl-*tert*-leucine chloroethylester

[1] For the isolation of the enzymatically formed *N*-acetyl-L-*tert*-leucine, the aqueous phase from dichloromethane extraction was brought to pH 1 by adding a concentrated HCl solution. The formed precipitate was removed by centrifugation and washed with water (90% chemical yield, 95.6% e.e.). The conversion of *N*-acetyl-L-*tert*-leucine to the free L-*tert*-leucine was performed as described above for D-*tert*-leucine.

(92.2 g, 92.2% chemical yield, >99.5% e.e.). This was easily converted to optically pure D-*tert*-leucine (83.8%) by heating with a 10% aqueous HCl solution, neutralizing the hydrochloride with a Dowex 1x4 ion exchanger (HO^- form), filtration and final evaporation of the filtrate.

A full description of the chemical synthesis of the racemic *N*-acetyl-*tert*-leucine chloroethylester (Fig 4b) can be found in [46]. This chemical synthesis and the enzymatic reaction were scaled up to the multi-kg level.

4. Chemo-enzymatic synthesis of rare and expensive inositol phosphates, including 5′-TAMRA-labeled (P1-tethered) D-PIP_3, using commercial lipase preparations

Chemo-enzymatic routes are also of great interest for the preparation of research biochemicals. This was demonstrated in our laboratory, e.g. for the preparation of inositol phosphates.

4.1. Importance of inositol phosphates as a tool for research

A significant number of physiological processes in differentiated higher cells are closely linked with inositol metabolism. Important examples are: the activation of thrombocytes in the blood clotting process, hormonal signal transduction, signal transformation, contraction of muscles, transmission and processing of neural signals, control of cell proliferation, bone biosynthesis and calcium metabolism, anchoring of proteins in membranes, etc. In order to gain a deeper insight into such cellular processes inositol phosphates, as important substrates for many of the involved enzymes, would provide a very elegant tool. However, only a limited selection of inositol phosphates is commercially available, in minute quantities and at very high costs. Most of the commercially available material is derived from organ preparations.

An easier access was searched which would open the way to large quantities of a broad variety of enantiomerically pure inositol phosphates. The chosen strategy was a chemo-enzymatic approach starting from *myo*-inositol and involving commercially available enzyme preparations for the key synthesis steps.

5′-TAMRA-labeled (P1-tethered)-D-*myo*-inositol-3,4,5-triphosphate (TAMRA-X-PIP_3) was required as a biochemical tool for binding assays in order to identify and characterize pharmaceutically relevant proteins having a 3,4,5-binding domain for phosphatidyl-D-*myo*-inositol-3,4,5-triphosphate (PI-(3,4,5)-P_3). TAMRA is a convenient and widely used fluorescence label.

4.2. Chemo-enzymatic syntheses of important inositol phosphates

D-*myo*-Inositol-1-phosphate (D-1-IP_1), our first target compound, was synthesized by a short and facile route from optically pure D-1-acetoxy-4,6-di-*O*-benzyl-*myo*-inositol, which was easily obtained by a highly regio- and enantioselective acylation of 4,6-di-*O*-benzyl-*myo*-inositol catalyzed by lipase PS from *Pseudomonas* sp. (Amano) followed by chemical phosphorylation and deprotection [48]. The key intermediate 4,6-di-*O*-benzyl-*myo*-inositol can easily be prepared from *myo*-inositol in four chemical steps. The benzyl protecting group allows the final deprotection under mild and neutral conditions without any P-migration.

D-1,3,4,5-IP_4 and the unnatural enantiomer L-1,3,4,5-IP_4 were prepared from D- and L-2,6-*O*-dibenzyl-*myo*-inositol by a chemical phosphorylation and deprotection step in high yields and purities without extensive purification. The diprotected racemic 2,6-di-*O*-benzyl-*myo*-inositol is an excellent precursor for the synthesis of racemic 1,3,4,5-IP_4 and can easily be prepared from *myo*-inositol in four chemical steps [49]. However, the enantioselective synthesis of D- or L-1,3,4,5-IP_4 requires the optically pure 2,6-di-*O*-benzyl-*myo*-inositols as building blocks. A classical chemical resolution procedure for racemic 2,6-di-*O*-benzyl-*myo*-inositol is so far unknown.

A screening of several commercially available lipases for their acyltransfer activity towards racemic 2,6-di-*O*-benzyl-*myo*-inositol in vinyl acetate as an acyl donor indicated that only the lipase from *C. antarctica* (Novo SP 435) offers reasonable conversion. HPLC and TLC analysis showed that only one product was formed and the rate of the reaction decreased dramatically after the conversion reached 50%, indicating a very high enantiospecificity. A preparative experiment was worked up after

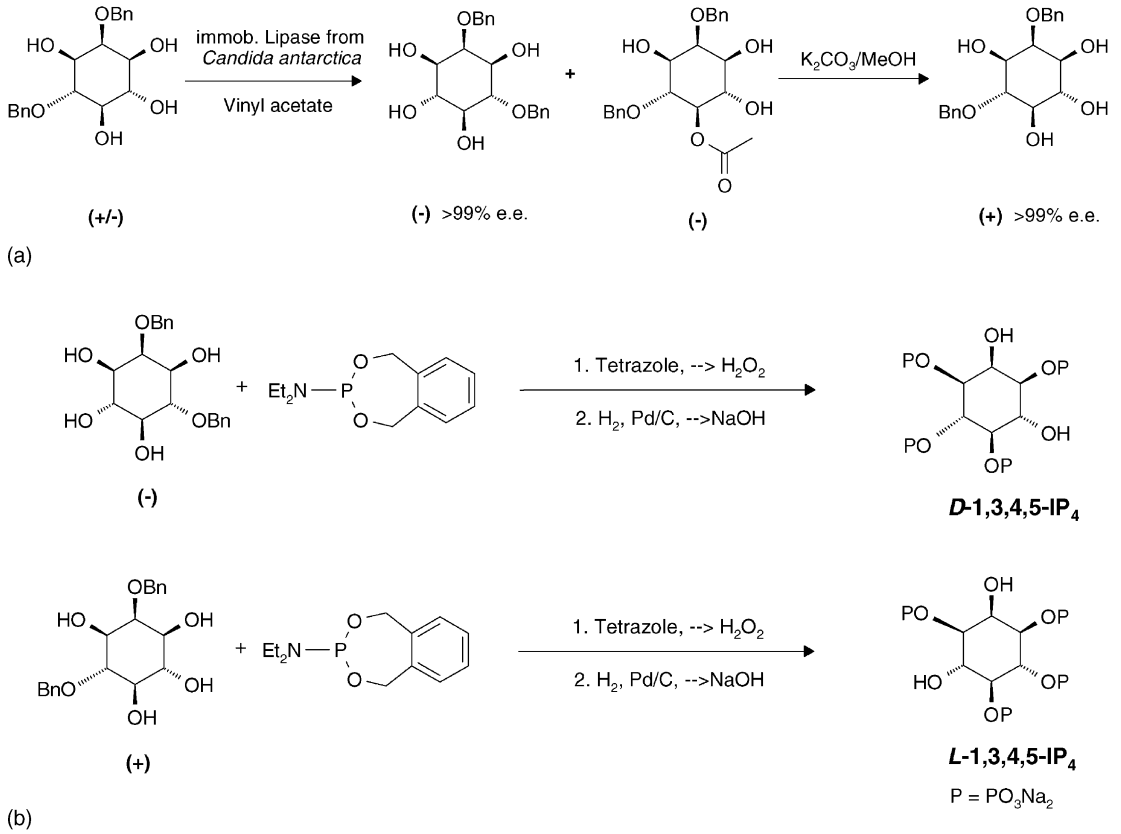

Fig. 5. (a) The enzymatic key step in the synthesis of D- and L-1,3,4,5-IP_4. (b) The phosphorylation and deprotection of the chiral key intermediates **(−)**- and **(+)**-2,6-di-*O*-benzyl-*myo*-inositol.

approximately 50% conversion yielding 49% (98% theoretical yield) of the unconverted inositol derivative **(−)**-2,6-di-*O*-benzyl-*myo*-inositol with an optical purity of >99 and 49% of monoacetylated derivative. Based upon the two-dimensional COSY and the ^{13}C NMR spectra, the monoacetate was isomerically pure and the hydroxyl group at C5 was esterified. Chemical hydrolysis of the monoacetate yielded quantitatively the **(+)**-2,6-di-*O*-benzyl-*myo*-inositol (>99% e.e.) [50] (Fig. 5a).

Both enantiomers of 2,6-di-*O*-benzyl-*myo*-inositol were finally phosphorylated using 2-di-ethylamino-1,3,2-benzo-dioxaphosphepane/tetrazole, followed by successive oxidation with H_2O_2. Deprotection with H_2-Pd/C removes all benzylic protecting groups leading to D- or L-1,3,4,5-IP_4, respectively [50] (Fig. 5b). Using the synthesis route described above 3.5 g of each D- and L-1,3,4,5-IP_4 as potassium salts (storage form) were synthesized.

By extending the strategies outlined above, a whole set of 12 different types of inositol phosphates could be made available in preparative scale starting from of 4,6- and 2,6-di-*O*-benzyl-*myo*-inositol [51]: D-IP_1, D/L-1,5-IP_2, D-1,5-IP_2, D-1,4,6-IP_3, D-2,3,5-IP_3, 1,2,3,5-IP_4, D/L-1,3,4,5-IP_4 = D,L-1,3,5,6-IP_4, D/L-1,4,5,6-IP_4 = D/L-3,4,5,6-IP_4, D-1,4,5,6-IP_4, D-1,3,4,5-IP_4, L-1,3,4,5-IP_4 = D-1,3,5,6-IP_4, and 1,3,4,5,6-IP_5. These inositol phosphate species have been made available for academic and industrial research via Alexis Corporation (Switzerland).

For the synthesis of 5′-TAMRA-labeled (P1-tethered)-D-*myo*-inositol-3,4,5-triphosphate (TAMRA-X-

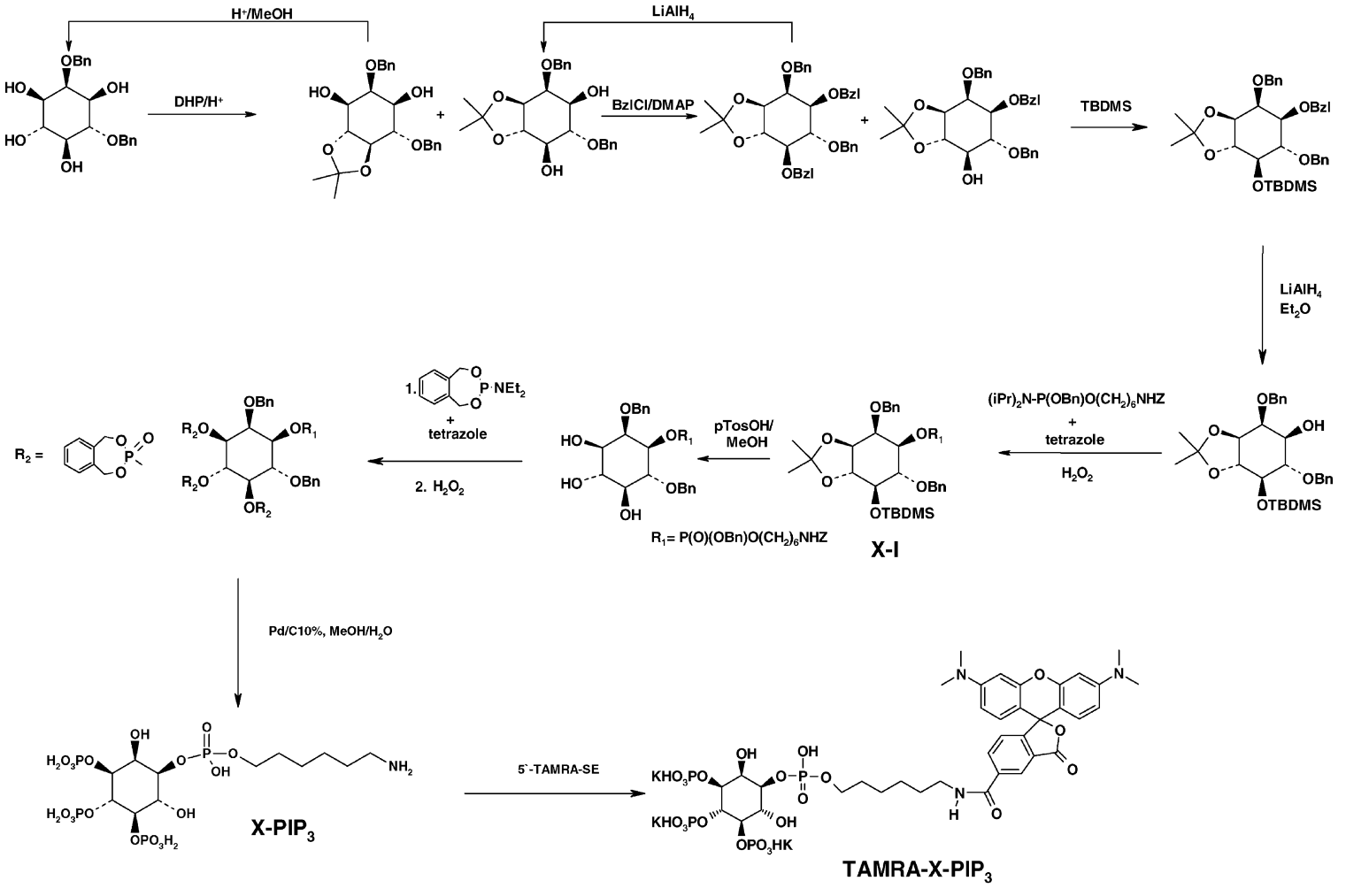

Fig. 6. The total synthesis of 5′-TAMRA-labeled (P1-tethered)-D-*myo*-inositol-3,4,5-triphosphate (TAMRA-X-PIP_3).

PIP_3) (Fig. 6) we again selected as starting material the optically pure D-(−)-2,6-di-*O*-benzyl-*myo*-inositol which was prepared by means of the lipase from *C. antarctica* (Novozym 435) as described above [50]. Reaction of D-(−)-2,6-di-*O*-benzyl-*myo*-inositol with 2,2-dimethoxypropane in CH_2Cl_2 (5 h, rt) with catalytic amounts of *p*-TosOH yielded as expected a 1:1 mixture of the 4,5- and 3,4-acetonides ($[\alpha]_D^{20} = -32.57^\circ$ ($c = 0.9$ MeOH) for the 3,4-acetonide) fortunately easily separable by column-chromatography. The unwanted 4,5-acetonide was recycled by treatment with *p*-TosOH in methanol and again converted. After three cycles, the 3,4-acetonide was obtained in an overall yield of 83%. Reaction of the D-(−)-2,6-di-*O*-benzyl-*myo*-inositol-3,4-acetonide with 1.3 eq. benzoylchloride/DMAP in CH_2Cl_2 (1 h, rt) gave the 1-monobenzoate ($[\alpha]_D^{20} = -107.49^\circ$ ($c = 0.85$ MeOH)) in 57% yield, besides the 1,5-dibenzoate and unreacted 3,4-acetonide. The isomeric 5-benzoate was not found. The D-1-benzoyl-2,6-di-*O*-benzyl-*myo*-inositol-3,4-acetonide reacted quantitatively with TBDMS-triflate and Hünigbase (CH_2Cl_2, rt, 1 h) to the now fully protected 5-*O*-TBDMS-derivative ($[\alpha]_D^{20} = -25.94^\circ$ ($c = 0.9$ MeOH)). Reductive deprotection ($LiAlH_4$, Et_2O) in position 1 (91% yield), phosphitylation and oxidation [52] gave the fully protected P1-tethered inositol (X-I) in 85% yield. Removal of the isopropyliden- and TBDMS-protecting group with *p*-TosOH in methanol (rt, 6 h) (84% yield, $[\alpha]_D^{20} = -35.91^\circ$($c = 0.8$ MeOH)), phosphorylation with 2-di-ethylamino-1,3,2-benzo-dioxaphosphepane and oxidation in analogy to the approach described earlier [50] (94% yield) followed by quantitative hydrogenolysis (Pd/C 10%, MeOH/H_2O (4:1)) of all benzyl groups afforded X-PIP_3. Coupling of the amino group with 5′-TAMRA-SE[2] under standard conditions (0.2 M potassium bicarbonate buffer, pH 8.3) followed by Sephadex G15 purification gave TAMRA-X-PIP_3 as potassium salt.

The synthesized TAMRA-X-PIP_3 proved to be fully functional in a protein binding assay designed for the pleckstrin homology domain of Cytohesin-1 and in a competition assay with D-1,3,4,5-IP_4 [53].

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