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Enzymatic Catalysis in Organic Synthesis.† Synthesis of Enantiomerically Pure Cα-Substituted α-Amino and α-Hydroxy Acids

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Enzymes are versatile catalysts in asymmetric synthesis. We have developed synthetic methodologies for the synthesis and resolution of α-amino amides (both α-H and α,α-disubstituted) using preparations of *Pseudomonas putida, Mycobacterium neoaurum* and *Ochrobactrum anthropi* with amidase and aminopeptidase activity. Also, highly substituted α,α-disubstituted amino acids and hydroxy acids can be obtained in enantiomerically pure form using pig liver esterase. In addition, an elegant kilogram-scale synthesis of D-malate has been developed using a maleate hydratase preparation from *Pseudomonas pseudoalcaligenes*. Finally, procedures are described for the synthesis of enantiomerically pure solketal and glycidol using, respectively, whole cell preparations of *Comamonas testosteroni* and *Bacillus pasteurinanus* containing the PQQ-dependent quinohaemoprotein ethanol dehydrogenase (QH-EDH).

The synthesis of α-amino acids remains a topic of considerable interest because of the ever growing importance of both natural and synthetic amino acids and derivatives. It is crucial that such compounds are available in enantiomerically pure form, because of the divergent biological activities of the enantiomers.

Optically pure α,α-disubstituted α-amino acids, especially α-methyl substituted amino acids[1–3] are of increasing interest for the agrochemical and pharmaceutical industry. More recently, higher disubstituted amino acids have also been described for this use.[4,5] These compounds may act as enzyme inhibitors and several are antagonists of receptors. Also the influence of these disubstituted amino acids on peptide structure and activity is of current interest.[6–8] In addition, enantiomerically pure (α-alkylated) α-hydroxycarboxylic acids are valuable synthetic intermediates for which only a limited number of efficient syntheses exist.[9]

We have developed several chemo-enzymatic approaches towards the synthesis of these classes of compound. In this review our results are summarized.

α-H and α,α-disubstituted amino acids

α-H amino acids; aminopeptidase from Pseudomonas putida *(ATCC 12633).* Of the several methods known to obtain α-amino acids as pure enantiomers, a particularly attractive method involves the use of an L-specific aminopeptidase, produced by a *Pseudomonas putida* strain (ATCC 12633), in an enzymatic kinetic resolution of a racemic mixture of α-amino acid amides. This enzymatic reaction (Fig. 1) can be applied successfully to a wide variety of amino acid amides.[10–12] The group R can vary widely, from CH_3, the amino acid alanine, to β-[(*E*)-6-(4-hydroxy-3-methylbut-2-enylamino)purin-9-yl] representing lupinic acid.[13]

In a continuing effort to broaden the scope of this versatile resolution we have developed new methodologies to synthesize more functionalized amino acid amides. One of the approaches involves the $BF_3 \cdot OEt_2$-mediated reaction of allylsilanes with *N*-(alkoxycarbonyl)-α-methoxy-

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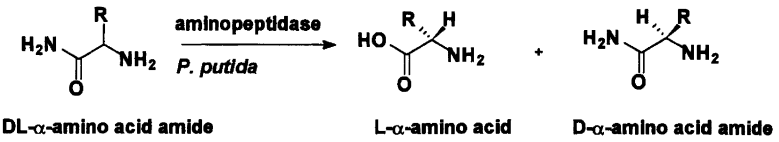


Fig. 1. Enzymatic resolution of α-H amino acid amides.

glycine esters **1**, and with (*N*-methoxy-)amides **2** and **3**, affording after deprotection the corresponding γ,δ-unsaturated α-aminocarboxy esters **5** and (*N*-methoxy) amides **6** and **7**, respectively (Fig. 2). A variety of allylsilanes have been used[14] in this reaction, including allyltrimethylsilane **4a**. 3-(trimethylsilyl)cyclohexene **4b** and 3-(trimethylsilyl)cyclopentene **4c**.

The α-aminocarboxamides **6** and **7** and their saturated derivatives could be successfully resolved using the aminopeptidase from *Pseudomonas putida* as the biocatalyst, indicating that in addition to the primary amides **6**, *N*-methoxycarboxamides **7** are also suitable substrates in this resolution process.

Similar to the reaction of allylsilanes with iminium intermediates **A**, silyl enol esters, like compound **8**, may also react with this type of versatile intermediates,[15] as indicated in Fig. 2. Although primary amides generating iminium intermediates **B**, are not suitable as substrates in the reaction with silyl enol esters, tertiary amides, like dimethyl substituted amides **10**, afford the corresponding γ-oxo-α-amino acid derivatives **13** and **14**, by $BF_3 \cdot OEt_2$ initiated reaction of silyl enol esters **11** and **12** (see Fig. 3).

Furthermore *N*-methoxy-*N*-trimethylsilyl substituted amides, like compound **15**, will react via C–C bond formation. Unexpectedly, a secondary reaction takes place to form 3-amino-2-pyrrolidinones **17** (with the phenyl-substituted silyl enol ether **11**) or 3-aminopyrrolinones **18** (after reaction with the *tert*-butyl silyl enol ether **16**), as shown in Fig. 4. Most probably, the products are formed via the intermediacy of a second iminium intermediate **C** formed after reaction of compound **15** with the silyl enol esters **11** or **16** to form **19**, and subsequent cyclization followed by a TMS-*N*,*O*-shift and elimination. Proton loss from the iminium intermediate **C** eventually furnishes the isolated 3-aminopyrrolinones **18**, whereas reaction of the cyclic iminium intermediate with a second molecule of the silyl enol ether will afford the observed 3-aminopyrrolidinones **17** (Fig. 5).[15]

Although a wide variety of amino acid derivatives are available via the resolution methodology using the *Pseudomonas putida* aminopeptidase preparation, it may be advantageous to synthesize enantiomerically pure functionalized amino acid derivatives via a common intermediate. Since allylglycine derivatives are readily available via the methodology discussed above, we have ex-

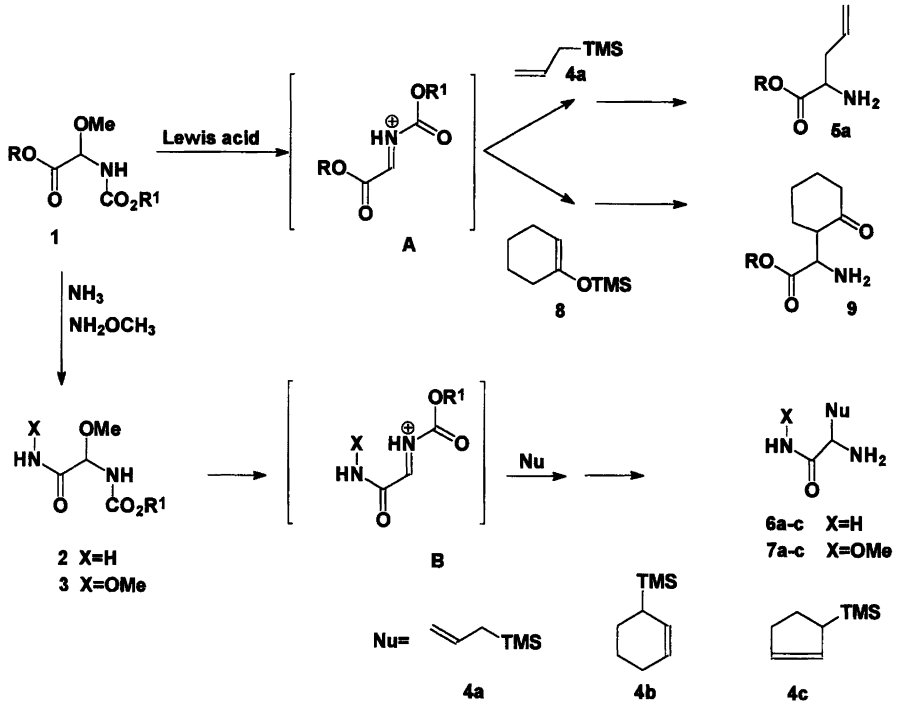


Fig. 2. Synthesis of functionalized amino acids via α-acyliminium intermediates.

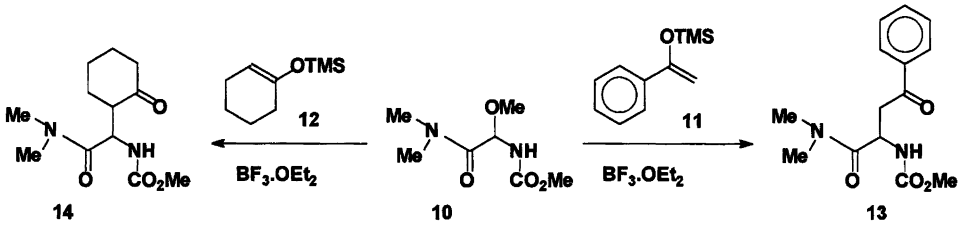


Fig. 3. Coupling of *N,N*-dimethyl-α-methoxyglycinamide derivatives with silyl enol ethers.

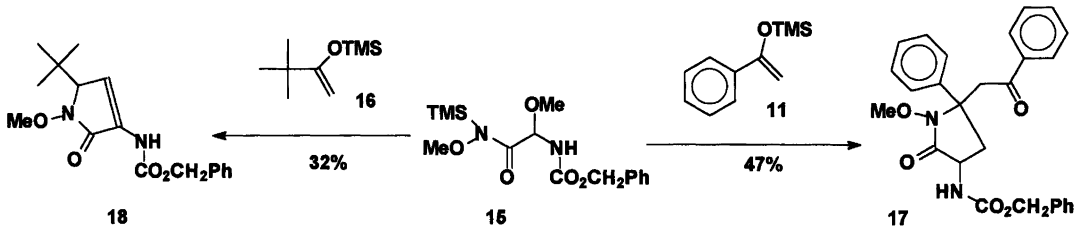


Fig. 4. Coupling of *N*-methoxy-*N*-trimethylsilyl-α-methoxyglycinamide derivatives with silyl enol ethers.

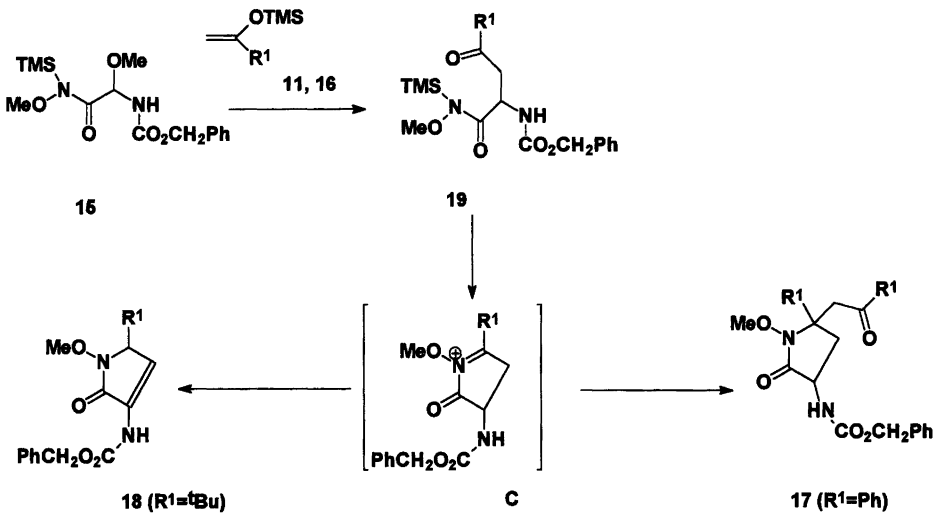


Fig. 5. Formation of 3-amino-2-pyrrolidinones and 3-amino-2-pyrrolinones.

plored the synthesis of optically active sulfur-containing trifunctional amino acids via radical addition to unsaturated amino acids and derivatives.[16] Indeed, it proved possible smoothly to add sulfur-based radicals, generated from R^1–S–H type precursors **20** (R^1 = alkyl, R^1 = acyl) with azoisobutyronitrile (AIBN), to α-allylglycine derivatives **21** protected at none, one or both of the amino acid functions (NH_2 and/or CO_2H). Sulfur-containing trifunctional amino acids **22** were obtained in good to excellent yields (64–100%). The reaction scheme is depicted in Fig. 6.

α,α-Disubstituted amino acids; amidase from Mycobacterium neoaurum *(ATCC 25795).* Because of the increasing interest in α,α-disubstituted amino acids a screening program was initiated to explore the possibility of using amidases in an enzymatic resolution of α,α-disubstituted amino acid amides. The first result of this screening was an amidase preparation from *Mycobacterium neoaurum* (ATCC 25795),[17] which could be used for the facile and large-scale preparation of a number of α-methyl substituted amino acids (Fig. 7) in which R could be alkyl or aryl.[18] Even sterically more demanding substrates such as α-ethyl- or α-allyl-phenylalanine amide could be resolved with this enzyme preparation.[19]

Despite the fact that in this resolution process the unwanted isomer could not be racemized and recycled directly – like in the analogous resolution of α-H amino acid amides – this resolution of disubstituted amino acid amides is attractive from the point of view of optical purity of the products and the ease of scaling-up to multikilogram quantities. Based on the resolution of a wide variety of substrates, a schematic model of the *Mycobacterium neoaurum* active site was proposed.[19] Biochemical

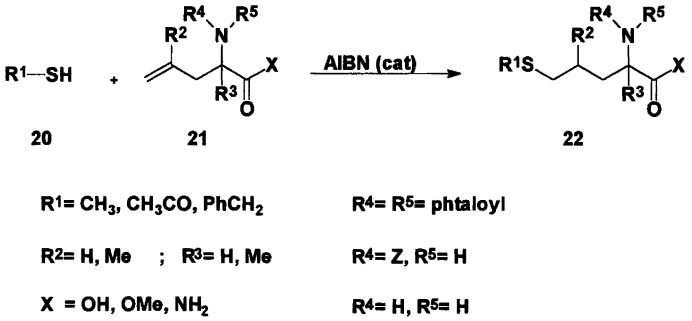


Fig. 6. Radical additions to unsaturated amino acid derivatives.

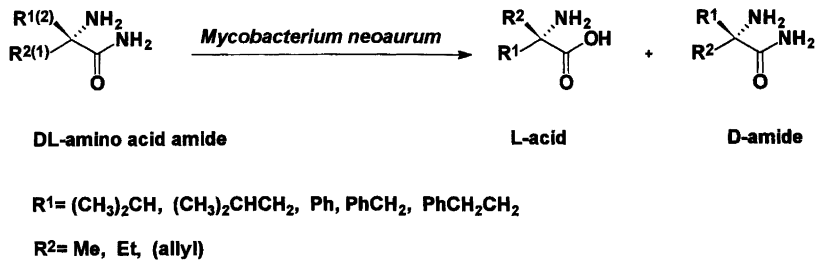


Fig. 7. Enzymatic resolution of α,α-disubstituted amino acid amides.

studies indicate that the amidase is a cysteine-containing hydrolase.[17] No substituents on the amino nitrogen are allowed, a small hydrophobic region which can contain the maximum of a C3-unit must be present. For the large substituent no constraints are observed, only direct attachment of an aromatic substituent to the chiral centre restricts the activity. This problem could be circumvented in part by the use of pig liver esterase[19] (see below). For the more hydrophobic substrates the solubility in water decreases, which is a major disadvantage of the system. A solution to this problem was eventually found by the use of a different amidase, produced by *Ochrobactrum anthropi*.[20] The latter preparation also proved to be quite useful in the resolution of sterically demanding amino acid amides (see below).

α,α-Disubstituted amino acids; pig liver esterase. From earlier studies[21,22] on the resolution of α-substituted α-hydroxy acids (see below) it is known that pig liver esterase can be used for the resolution of highly substituted compounds, such as α-phenyl-α-allyl-α-hydroxyacetic acid ethyl ester **41** (Fig. 12). Therefore we embarked on the resolution of analogous α,α-disubstituted amino acid esters with pig liver esterase (source: Amano). Interestingly, the enantioselectivity of the esterase proved to be complementary to the enantioselectivity of the amidase from *Mycobacterium neoaurum*. Some results of the resolutions are summarized in Table 1.

Pig liver esterase showed low enantioselectivity in the resolution of α-methyl-α-phenyl-glycine ethyl ester **23** (X = OEt) or α-ethyl-α-phenylglycine ethyl ester **24** (X = OEt). In contrast, *Mycobacterium neoaurum* amidase can be used in the resolution of compounds **23** and **24** (X = NH_2) with high enantioselectivity. However, rates and yields are rather low. Remarkably, the pig liver esterase catalyzed resolution of α-allyl-α-phenylglycine ethyl ester **25** (X = OEt), and α-butyl-α-phenylglycine ethyl ester **26** (X = OEt) proceeded with a satisfactory enantiomeric ratio (*E*), whereas the corresponding amides **25** and **26** (X = NH_2) were not substrates for the *Mycobacterium neoaurum* amidase. However, these amides could readily be resolved using the amidase from *Ochrobactrum anthropi*[20] (see below).

α,α-Disubstituted amino acids; amidase from Ochrobactrum anthropi *(NCIMB 40321).* Although the amidase from *Mycobacterium neoaurum* proved to be quite effective in the synthesis of enantiomerically pure α,α-disubstituted amino acids, which were not too sterically demanding at the chiral carbon atom, the system is not ideal for more heavily substituted amino acids. However, after an intensive screening program we were able to isolate a new amidase preparation from an *Ochrobactrum anthropi* strain (NCIMB 40321).[20] This amidase preparation proved to be highly versatile: not only can this enzyme preparation be used for the resolution of α-H amino acids with high stereoselectivity, the amidase also resolved a very broad range of sterically highly demanding disubstituted amino acid amides, including α-phenyl-α-alkyl disubstituted amino acid amides **25** and **26** (X = NH_2, see Table 1). Remarkably, the enzyme preparation could also be used for the resolution of α-hydroxy acid derivatives such as mandelic acid amide and for the resolution of *N*-hydroxyamino acid amides, making it a far more versatile biocatalyst than the *Mycobacterium neoaurum* amidase. The amidase from *Ochrobactrum anthropi* also

Table 1. Enzymatic resolution of α,α-disubstituted α-amino acid esters and amides.

	H_2N COX Ph Me		H_2N COX Ph Et		H_2N COX Ph		H_2N COX Ph	
	X=OEt	$X+NH_2$	X=OEt	$X=NH_2$	X=OEt	$X=NH_2$	X=OEt	$X=NH_2$
Conversion (%)	14	48[a]	66	13[b]	57	–[c]	51	–[c]
(*R*)-substrate								
chem. yield (%)	67	42	28	59	41	–	31	–
% ee	5	86	25	14	95	–	97	–
(*S*)-acid								
chem. yield (%)	6	35	40	9	57	–	41	–
% ee	31	95	13	94	72	–	93	–
E[d]	2	110	2	50	23	–	114	–

[a] Reaction time, 48 h. [b] Reaction time, 7 days. [c] No reaction observed. [d] *E*=enantiomeric ratio.

showed a remarkably broad pH-optimum. The optimum pH is about 8.5 but at pH 5 the enzyme still has about 50% of its activity. This latter property makes this enzyme extremely useful in the resolution of hydrophobic amino acid amides, since, owing to the presence of the amino-function, solubility increases at lower pH values. An example can be found in the resolution of α-methyl(3,4-dichlorophenyl)alanine amide **29**, a useful precursor in the synthesis of enantiopure cericlamine **32**, a novel potent and selective synaptosomal 5-HT uptake inhibitor developed by Jouveinal. The process is depicted in Fig. 8. In the resolution step the pH was 5.5. The resulting (*S*)-amino acid **30** was further transformed into the desired product via reduction of the amino acid using $NaBH_4$–H_2SO_4, to form the amino alcohol **31**, followed by an Eschweiler–Clarke methylation,[23] affording the desired cericlamine **32**.

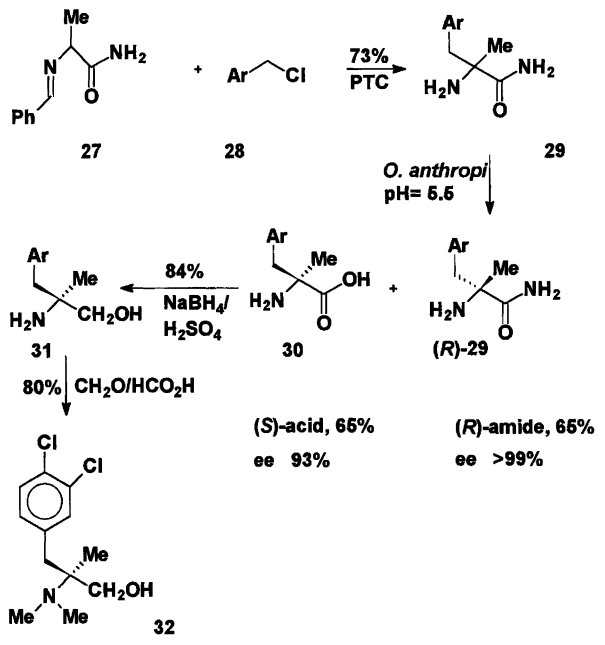


Fig. 8. Synthesis of (*S*)-cericlamine.

The efficient large-scale synthesis of amino alcohols from the corresponding amino acids or derivatives is still a challenging task. Recently we developed a new method[24] for the transformation of amino acid amides into amino alcohols by a dissolving metal reduction (sodium in refluxing propanol). Although considerable racemisation was observed for α-monosubstituted α-amino acid amides, α,α-disubstituted α-amino acid amides were reduced without any racemisation. This method is especially useful for the synthesis of (*R*)-disubstituted amino alcohols from the corresponding (*R*)-α,α-disubstituted amino acid amides; the remaining products in the resolution processes are as described above.

Synthesis of racemic α,α-disubstituted amino acid amides and esters. In the resolution processes described above, α,α-disubstituted amino acid amides are required as the substrates, necessitating the availability of efficient synthetic routes to these starting materials. At this point it is appropriate to note that, in principle, asymmetric synthesis of α,α-disubstituted amino acids appears to be the method of choice – compared with a resolution process – since in the latter the unwanted isomer cannot be simply racemized and recycled, thereby limiting the overall yield to a maximum of 50%. However, asymmetric synthesis of these types of compound are often laborious and difficult to perform on a large scale, whereas the enzymatic resolution process can easily be performed on the kilogram scale.

An interesting approach towards the synthesis of disubstituted amino acid esters by phase-transfer catalyzed alkylation has been described by O'Donnell.[25] Since in the enzymatic resolution process disubstituted amino acid amides are used as substrates, we attempted to convert the disubstituted amino acid esters, products of the O'Donnell procedure, into the corresponding amides, using aqueous or methanolic ammonia. This standard pro-

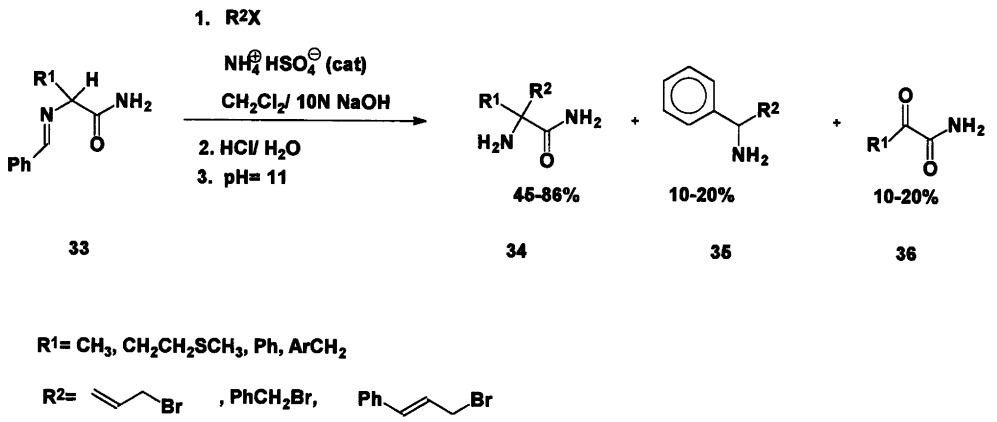


Fig. 9. Synthesis of disubstituted amino acid amides via phase-transfer alkylation.

cedure, however, was not always successful in the case of sterically highly demanding substrates. Therefore, we developed an alternative method of preparation of the desired racemic disubstituted amino acid amides using a modified O'Donnell procedure.[26]

N-Benzylidene α-H α-amino acid amides **33**, readily available from benzaldehyde and α-amino acid amides, can be alkylated at room temperature in 4–18 h under phase-transfer conditions in CH_2Cl_2–10 M NaOH solution using tetrabutylammonium hydrogensulfate as the phase-transfer catalysts (Fig. 9). Weakly acidic hydrolysis (during the work-up procedure) afforded the α,α-disubstituted α-amino acid amides **34** in 45–86% yield. Remarkably, no amide alkylation was observed. In addition to the desired α-alkylation, some 10–20% alkylation at the imine carbon occurred, eventually resulting in the formation of compounds **35** and **36**. High alkylation yields were obtained from Schiff bases of amino acid amides and 1 equivalent of an activated alkyl halide (allyl bromide, cinnamyl bromide, benzyl bromide). With sterically hindered amino acid amides yields were lower. In the case of the Schiff base of valine amide no alkylation occurred. Less activated (primary) alkyl halides afforded moderate yields and with secondary alkyl halides only elimination was observed. This procedure was also used in the synthesis of cericlamine **32**, starting from the Schiff base of alanine amide **27** and 3,4-dichlorobenzyl chloride **28**. The desired racemic α,α-disubstituted acid amide **29** was isolated in a yield of 73% (Fig. 8).

Since in the asymmetric phase-transfer catalyzed alkylation of glycine derivatives enantiomeric excesses up to 66% have been obtained,[27] it appeared attractive also to attempt asymmetric phase-transfer catalyzed alkylation reactions to form disubstituted amino acid derivatives. Unfortunately, however, the use of benzylated cinchona alkaloids as phase-transfer catalysts in the allylation of *N*-benzylidene phenylglycine ethyl ester in CH_2Cl_2–10 M NaOH gave very low inductions. However, recently, O'Donnell *et al.*[27] reported a slightly modified procedure for the synthesis of disubstituted amino acid *tert*-butyl esters with ee values of up to 50%.

Another approach towards the synthesis of function-

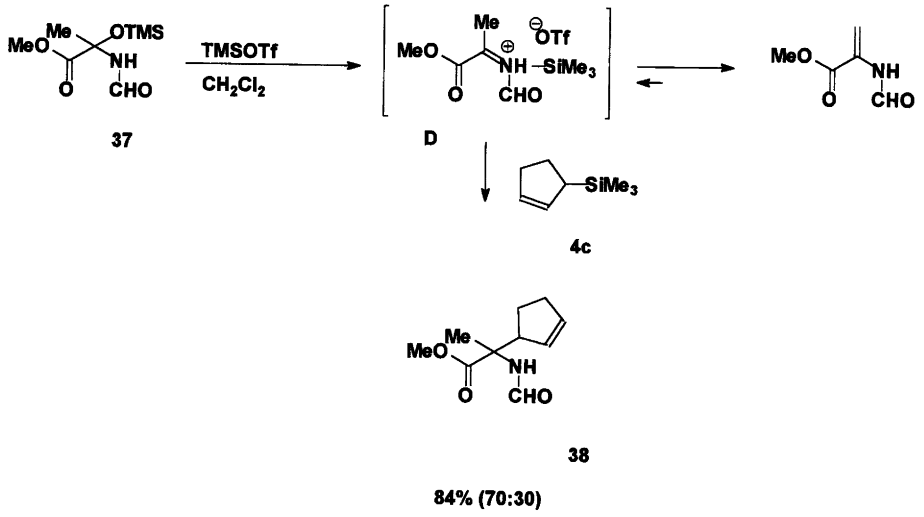


Fig. 10. Synthesis of γ,δ-unsaturated α-Me-α-amino acids via coupling of allylsilanes with an α-methylglycine cation equivalent.

alized disubstituted amino acids involves the coupling of allylsilanes with an α-alanine cation equivalent,[28,29] affording γ,δ-unsaturated α-methyl-α-amino acids. *N*-Formyl-2-trimethylsilyloxyalanine methyl ester **37** was used as the α-alanine cation equivalent, the formation of the cation **D** was induced by trimethylsilyl trifluoromethanesulfonate. As an example the formation of *N*-formyl-α-methyl-α(3-cyclopentenyl)glycine methyl ester **38** is depicted in Fig. 10.

α-H and α,α-disubstituted α-hydroxy acids and derivatives

α-H hydroxy acids; maleate hydratase from Pseudomonas pseudoalcaligenes *(NCIMB 9867).* As mentioned above, the *Ochrobactrum anthropi* amidase preparation could be used for the resolution of e.g., mandelic acid amides.[20] An intrinsic disadvantage of such a resolution process is the formation of a product mixture and the need to racemize and recycle the unwanted isomer. A conceptually more elegant approach is the asymmetric synthesis of α-hydroxy acids starting from prochiral substrates. For example, L-malate has been produced since 1974 in a continuous process that uses immobilized *Brevibacterium flavum* cells containing high fumarase activity.[30] We have explored the possibility of producing D-malate using a similar approach.[31–35]

After an extensive screening program a *Pseudomonas pseudoalcaligenes* strain was found[31,32] which converted maleate into D-malate in a yield of 99.4%.[33] The optical purity of the product was over 99%. *P. pseudoalcaligenes* NCIMB 9867 was grown at 30°C in a mineral salt medium with 3-hydroxybenzoate as the carbon source. The cells were harvested during the log-phase, washed and permeabilized. The maleate hydratase reaction is cofactor- and oxygen-independent and the bioconversion can be performed in a stirred vessel. The enzyme activity is strongly affected by the maleate salt used as the substrate.[34] With Na_2-maleate as the substrate, maleate hydratase activity decreased strongly with increasing substrate concentrations. By using counter-ions other than sodium, the concentrations of available dissociated maleate could be reduced significantly, owing to metal–substrate complex formation (Fig. 11). Use of counter-ions that resulted in the formation of an insoluble metal–substrate complex, such as Ca^{2+} (compound **39**), further reduced the maleate concentration. Using this counter-ion, a crystal–liquid two-phase system is formed,[34] because the metal–product complex **40** also has a very low solubility. In this manner high yields of product could be obtained in high concentrations (over 160 g^{-1}). Using this system, 1 kg of D-malate could be produced in a stirred 5-l Erlenmeyer within 48 h, using 1 $g\,l^{-1}$ of protein preparation (whole cells).[35]

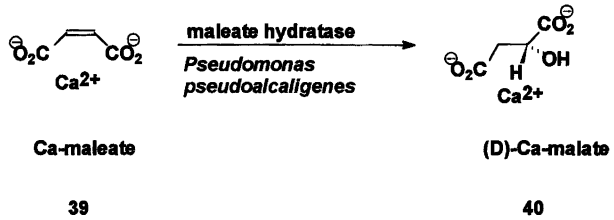


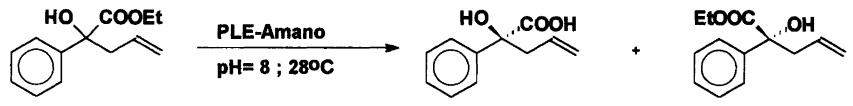
Fig. 11. Formation of enantiomerically pure D-malate from maleate.

α,α-Disubstituted α-hydroxy acids. For the synthesis of enantiomerically pure α,α-disubstituted α-hydroxy acids two approaches were followed. Initially, we explored the Pd-catalyzed allylation of 1,3-dioxolan-4-ones (synthesized from mandelic or lactic acids) to form allylated dioxolanones, followed by hydrolysis to the corresponding α-allyl-α-hydroxy acids.[36] Although a catalytic process could be developed for the synthesis of racemic material, the asymmetric variant, however, did not lead to optically pure products. The enantiomeric excess did not exceed 30%. Alternatively, pig liver esterase catalyzed resolution of α-allyl-α-phenyl-α-hydroxyacetic acid ethyl ester **41** (Fig. 12) afforded in a relatively facile process multi-gram quantities of optically pure products (*S*)-**42** and (*R*)-**42** (after recrystallization).[21,22]

Enantioselective oxidation of 2,2-dimethyl-1,3-dioxolan-4-ylmethanol (solketal) and 2,3-epoxy-1-propanol (glycidol); quinohaemoprotein ethanol dehydrogenase. Enantiomerically pure glycidol (*R*)-**43**, and solketal (*S*)-**45** are attractive C_3-synthons for the synthesis of various homochiral pharmaceuticals. Several approaches towards the synthesis of these compounds are known including enzymatic resolution processes. Generally speaking, most of the proposed routes are rather complicated and/or use rather expensive starting materials, making them unattractive for commercialization. In view of the fact that both glycidol **43** and solketal **45** are relatively inexpensive, we envisaged an enantioselective oxidation as the basis for a simple and hopefully economically feasible process for obtaining the pure enantiomers.

The principle is depicted in Fig. 13. The crucial step is an enantioselective oxidation using whole cell preparations containing the PQQ-dependent quinohaemoprotein ethanol dehydrogenase.

(*S*)-2,2-Dimethyl-1,3-dioxolan-4-ylmethanol [(*S*)-solketal, (*S*)-**45**] could be obtained in enantiomerically pure form via oxidation of the racemic mixture to form the corresponding (*S*)-acid, by cells of *Comamonas testosteroni*[37] LMD 26.36 supplemented with PQQ (pyrroloquinolinequinone). The enantioselectivity of the oxidation had an *E* value (enantiomeric ratio) of 49. The strain was grown on ethanol. The quinohaemoprotein ethanol dehydrogenase (QH-EDH) was produced in its *apo*-form. In principle the production of active cells can be integrated with the bioconversion step into one process, provided that PQQ and solketal **45** addition occur at the appropriate moment. However, further investigations are still needed to optimize this process.



41 42 41

(*S*)-acid (*R*)-ester

conversion (%)	cy	%ee	cy	%ee	E
34	29	94	52	49	52
51	44	83	41	86	30

Fig. 12. Pig liver esterase catalyzed hydrolysis of racemic α-hydroxy-esters.

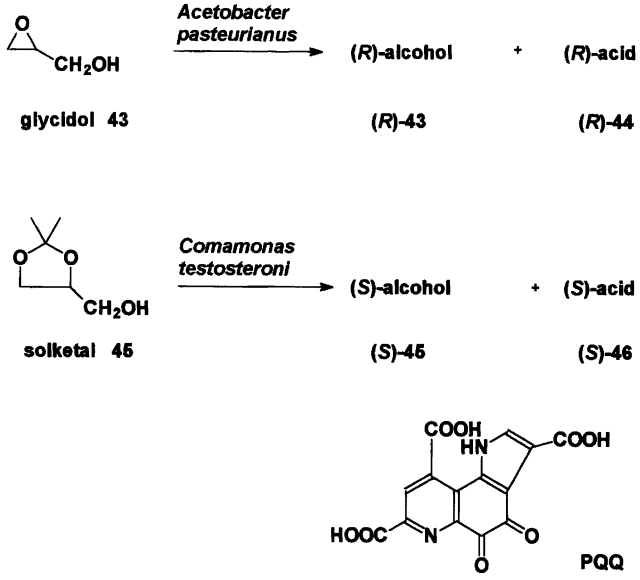


Fig. 13. Enantioselective oxidation of solketal and glycidol.

(*R*)-Glycidol [2,3-epoxy-1-propanol, (*R*)-**43**)] can be obtained from racemic glycidol by enantioselective oxidation with *Acetobacter pasteurianus* ATCC 12874.[38] This organism oxidizes glycidol **43** with an activity comparable to the oxidation of ethanol and has a preference for the (*S*)-enantiomer. This kinetic resolution process obeys a simple relationship, indicating an enantiomeric ratio *E* of 19. Eventually, *R*-glycidic acid **44** is formed, although there is a transient formation of the intermediate glycidaldehyde. Also in this process the PQQ-dependent quinohaemoprotein ethanol dehydrogenase (QH-EDH) is responsible for the enzymic conversion.

Conclusion

Several practical enzymatic approaches towards the synthesis of enantiomerically pure highly substituted α-amino acid and α-hydroxy acid derivatives have been developed. A set of amidases and aminopeptidases, produced by *Pseudomanas putida, Mycobacterium neoaurum* and *Ochrobactrum anthropi* strains is now available for the large scale production of a wide variety of amino acid derivatives. In special cases pig liver esterase can also be used for the resolution of sterically highly demanding substrates.

Pig liver esterase can also be used for the synthesis of several α,α-disubstituted α-hydroxy acids. In addition to these resolution processes using hydrolytic enzymes, two other approaches towards the synthesis of α-hydroxy acid derivatives have been explored. D-Malate can be produced on a kilogram scale in a very simple procedure with the cofactor- and oxygen-independent maleate hydratase from *Pseudomonas pseudoalcaligenes*. Finally, enantioselective oxidations with whole cell preparations containing the PQQ-dependent quinohaemoprotein ethanol dehydrogenase (from *Comamonas testosteroni* or *Acetobacter pasteurianus*) can be used for the synthesis of optically pure solketal (*S*)-**45** and glycidol (*R*)-**43**, valuable C_3-synthons.

We are continuing our efforts to find new enzymic resolution processes, using the enzymes discussed in this review, and others. An example of the latter is the chymotrypsin-mediated resolution of methyl 3-chloromethyl-2-tetrahydrofurancarboxylate.[39] Also, we are extending the applications of enantiomerically pure amino acid and hydroxy acid derivatives, e.g., as ligands in asymmetric homogeneous catalysis[40] or in asymmetric $NaBH_4$ reductions of cyclic imides.[41]

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