

Enzymatic Protecting Group Techniques[†]

Herbert Waldmann* and Dagmar Sebastian

Institut für Organische Chemie, Universität Karlsruhe, 76128 Karlsruhe, Germany

Received June 23, 1993 (Revised Manuscript Received March 8, 1994)

Contents

1. Introduction	911
2. Protection of Amino Groups	912
2.1. N-Terminal Protection of Peptides	912
2.2. Protection of the Side-Chain Amino Group of Lysine	914
2.3. Protection of Amino Groups in β -Lactam Chemistry	915
2.4. Protection of Amino Groups of Nucleosides	916
3. Protection of Thiol Groups	916
3.1. Protection of the Side-Chain Thiol Group of Cysteine	916
4. Protection of Carboxy Groups	916
4.1. C-Terminal Protection of Peptides	916
4.2. Protection of the Side-Chain Groups of Glutamic and Aspartic Acid	920
5. Protection of Hydroxy Groups	921
5.1. Protection of Carbohydrates	921
5.1.1. Monosaccharides	921
5.1.2. Di- and Oligosaccharides	927
5.1.3. Nucleosides	928
5.1.4. Further Aglycon Glycosides	929
5.2. Protection of Polyhydroxylated Alkaloids	931
5.3. Protection of Steroids	931
5.4. Protection of Phenolic Hydroxy Groups	933
5.5. Protection of Glycerol Derivatives and Related Polyols	933
6. Outlook	935
7. References	935

1. Introduction

The proper introduction and removal of protecting groups is one of the most important and widely carried out synthetic transformations in preparative organic chemistry. In particular, in the highly selective construction of complex, polyfunctional molecules, e.g. nucleotides, oligosaccharides, peptides, and conjugates of these natural products, as well as in the synthesis of alkaloids, macrolides, polyether antibiotics, prostaglandins and further natural products, regularly the problem arises that a given functional group has to be protected or deprotected selectively under the mildest conditions and in the presence of functional groups of similar reactivity, as well as in the presence of structures being sensitive to acids, bases, oxidation, and reduction. A representative example is given by the chemistry of the biologically rather important lipo-, phospho-, nucleo-, and glycopeptides (1-4, respectively) (Chart 1).

[†] Dedicated to Professor Dr. Helmut Ringsdorf on the occasion of his 65th anniversary.



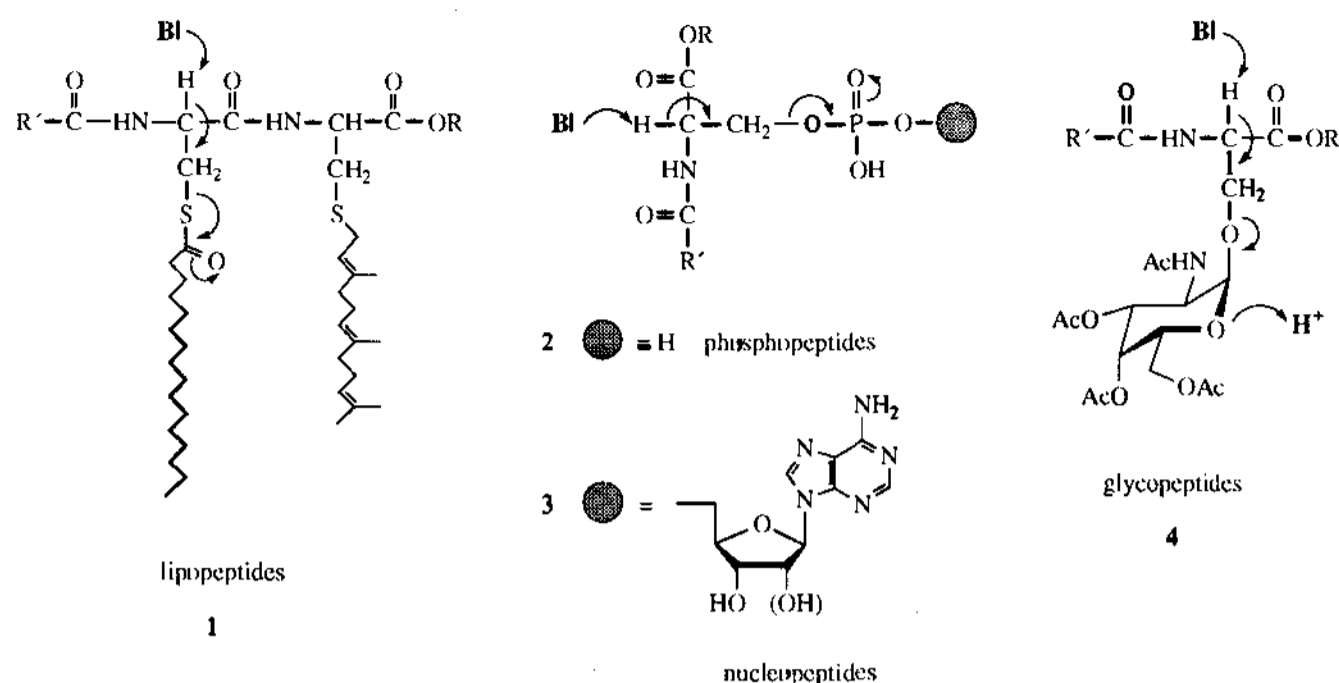
Herbert Waldmann was born in Neuwied, Germany, in 1957. He studied chemistry at the Johannes-Gutenberg-Universität Mainz and received his Dr. rer. nat. in 1985 under the guidance of Horst Kunz. After postdoctoral studies from 1985 to 1986 with George Whitesides at Harvard University he returned to the Universität Mainz and received his habilitation in 1991. After holding a professorship at the Rheinische Friedrich-Wilhelms-Universität Bonn from 1991 to 1993, he moved to the Universität Karlsruhe where he now is Full Professor of Organic Chemistry. Herbert Waldmann has been the recipient of the Friedrich Weygand award for the advancement of peptide chemistry and of the Carl Duisberg award from the Gesellschaft Deutscher Chemiker. His current research interests include the application of biocatalysts in organic synthesis, in particular for the construction of complex peptide conjugates, the development of new methods for diastereo- and enantioselective synthesis, alkaloid chemistry, and peptide and carbohydrate chemistry.



Dagmar Sebastian was born in Bad Neuenahr, Germany, in 1966. She studied chemistry at the Rheinische Friedrich-Wilhelms-Universität in Bonn and received her diploma degree in 1992. She then joined the research group of Professor H. Waldmann, and currently she is a graduate student at the University of Karlsruhe. In her graduate studies she concentrates on the development of chemoenzymatic techniques for the synthesis of phosphopeptides.

For the successful construction of these polyfunctional compounds, the hydroxy groups of the carbohydrates, the amino groups of the amino acids and the nucleotides,

Chart 1



the carboxy groups and the side chain functional groups of the amino acids, and the internucleotide phosphates have to be masked with blocking functions which are orthogonally stable to each other, i.e. which can be removed selectively leaving all other protecting groups intact. This challenge is further enhanced by the pronounced chemical lability of the peptide conjugates 1-4. Thus, on the one hand already under weakly basic conditions ($\text{pH} > 9$) the entire fatty acid, carbohydrate, nucleotide, and phosphate parts are cleaved off from 1-4. On the other hand, under acidic conditions an attack on the N- or O-glycosidic bonds in 3 and 4 may occur, resulting in an anomerization or even a complete decomposition of the glycosides.

For the manipulation of protecting groups under mildest conditions, numerous classical chemical methods have been developed.^{1,2} Nevertheless, there still remain severe problems during the synthesis of complex, polyfunctional molecules which cannot or only with great difficulties be solved by using classical chemical tools only. However, the arsenal of the available protecting group techniques has been substantially enriched by the application of biocatalysts. In addition to their stereodiscriminating properties, enzymes offer the opportunity to carry out highly chemo- and regioselective transformations. They often operate at neutral, weakly acidic, or weakly basic pH values and in many cases combine a high selectivity for the reactions they catalyze and the structures they recognize with a broad substrate tolerance. Therefore, the application of these biocatalysts to effect the introduction and/or removal of suitable protecting groups offers viable alternatives to classical methods.^{3,4}

2. Protection of Amino Groups³⁻⁵

2.1. N-Terminal Protection of Peptides

The selective protection and liberation of the α -amino function, the carboxy group, and the various side-chain functional groups of polyfunctional amino acids constitute some of the most fundamental problems in peptide chemistry. Consequently, numerous efficient protective functions based on chemical techniques were developed to a high level of practicability.^{1,2,6} However, since the mid-1970s, a systematic search for blocking

groups being removable with a biocatalyst has been carried out.³⁻⁵ In addition to the mild deprotection conditions they promise, protecting groups of this type are expected to be particularly useful for the construction and manipulation of larger peptide units, i.e. for transformations which, for solubility reasons, in general have to be carried out in aqueous systems. Also applications in the reprocessing of peptides obtained by recombinant DNA technology are foreseen.

Initial attempts to introduce an enzyme-labile amino protecting group involved the use of chymotrypsin for the removal of *N*-benzoylphenylalanine (Bz-Phe) from the tripeptide Bz-Phe-Leu-Leu-OH.⁷ The desired dipeptide H-Leu-Leu-OH was obtained in 80% yield under mild conditions (pH 7.3, room temperature). Chymotrypsin, however, is an endopeptidase of rather broad substrate tolerance, catalyzing the hydrolysis of peptide bonds on the carboxy groups of hydrophobic and of aromatic amino acid residues. Since such amino acids appear widely in peptides and no method is available to protect them against attack by the enzyme during the attempted deprotection, the use of chymotrypsin is problematic. It will, therefore, be limited to special cases⁸ in which no danger of competitive cleavage at undesired sites has to be feared. A protease of much narrower specificity is trypsin which catalyzes the hydrolysis of peptide bonds at the carboxylic group of lysine and arginine. These amino acids carry polar, chemically reactive side-chain functional groups which can be masked by various techniques.⁶ The sharp specificity of trypsin together with the possibility to hide the critical amino acids which function as primary points of tryptic cleavage allowed for the development of a broadly applicable system for the protection of the N-terminus of peptides.^{5,9-11} In several studies the application of trypsin-labile protecting groups, along with suitable protecting functions for the side chains of arginine and lysine were described.⁹⁻¹⁵ Thus, for instance Z-Arg-OH served as an enzymatically removable blocking group in a stepwise synthesis of de-aminoxytocin 6^{10,11} (Figure 1).

Starting with the pentapeptide 5 the amino acid chain was elongated with Z-Arg-protected amino acid *p*-nitrophenyl esters. The N-terminal Z-Arg protecting group was successively removed in moderate to high yields and without attack on the other peptide bonds

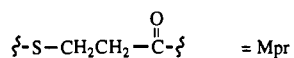
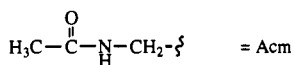
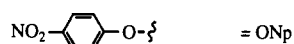
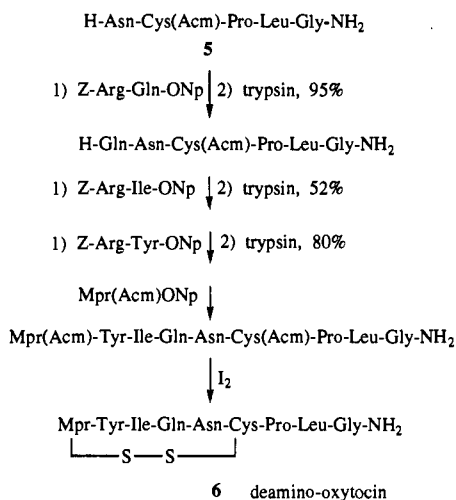


Figure 1.

by treatment with trypsin. Unfortunately, the preparation of the protected arginine *p*-nitrophenyl esters is difficult, thus preventing this method from becoming generally useful for the stepwise assembly of larger peptides. The trypsin-labile blocking groups have, however, proven to be very useful for the construction of oligo- and polypeptides via condensation of preformed peptide fragments. An illustrative example which consists of a chemoenzymatic construction of the 21–31 fragment 7 of murine epidermal growth factor is given in Figure 2. In the course of this synthesis the deblocking by trypsin was applied twice.⁸ The enzyme first liberated the N-terminus of a tetrapeptide and subsequently of a heptapeptide. In a synthesis¹⁶ of human β -lipotropin an Ac-Arg residue was introduced by a solid-phase technique at the N-terminus of the 29 C-terminal amino acids of the desired polypeptide. After cleavage from the resin and protection of the side-chain functional groups with citraconic acid, the arginine moiety was removed with trypsin, leaving the peptide chain intact. Finally, coupling of the citraconylated 61–89 fragment, thus obtained, to the partially protected 1–60 segment and subsequent deprotection delivered β -lipotropin, i.e. a polypeptide consisting of 89 amino acids. Further examples are found in syntheses of oxytocin,⁵ Met-enkephalin¹⁷ (vide infra, Figure 10), and Glu⁴-oxytocin.⁵

In addition to chymotrypsin and trypsin, the collagenase from *Clostridium histolyticum* has been proposed as a catalyst for the removal of N-terminally attached dummy amino acids from peptides.¹⁸ The enzyme recognizes the tetrapeptides Pro-X-Gly-Pro and splits the X-Gly bond. The use of this biocatalyst permitted the construction of des-pyroglutamyl-[15-Leu]human little gastrin I by selective hydrolysis of

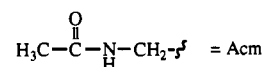
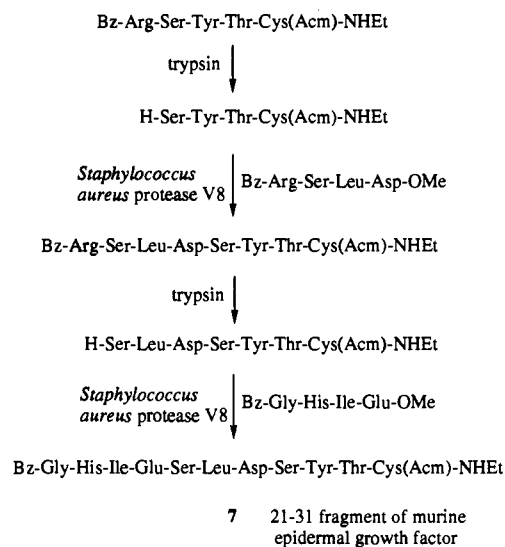
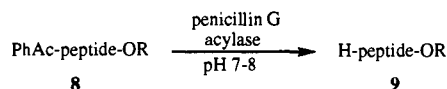


Figure 2.

the dipeptide Pz-Pro-Leu (Pz = 4-phenylazobenzyl-oxycarbonyl) from the N-terminus of the octadecapeptide Pz-Pro-Leu-Gly-Pro-Trp-Leu-(Glu)₅-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-NH₂. Transformations of this type resemble an analogy to the naturally occurring conversion of prohormones into hormones and may prove to be useful for the processing of peptide factors produced by recombinant DNA technology.

Despite the impressive syntheses that have been realized using proteases, the use of these enzymes is always accompanied by the danger of a competitive (and sometimes unexpected and unforeseeable) cleavage of the peptide backbone at an undesired site. At least, complex protecting group schemes may become necessary if the amino acid, which serves as recognition structure for the protease, occurs several times in the peptide chain to be constructed. This disadvantage can be overcome if a biocatalyst devoid of peptidase activity is used for the liberation of the N-terminal amino group. The principle has been realized by applying penicillin G acylase from *Escherichia coli*^{19–33} which is used in industry for the large-scale synthesis of semisynthetic penicillins (vide infra, Figure 6). This enzyme attacks phenylacetic acid (PhAc) amides and esters but does not hydrolyze peptide bonds. The acylase accepts a broad range of protected dipeptides 8 as substrates and selectively liberates the N-terminal amino group under almost neutral conditions (pH 7–8, room temperature), leaving the amide bonds as well as the C-terminal methyl, allyl, benzyl, and *tert*-butyl esters unaffected^{20–27,30} (Figure 3). In this transformation considerable amounts of organic cosolvents are tolerated. The PhAc group is easily introduced into amino acids by chemical³⁴ or enzymatic^{30,35} methods. It is stable during the removal of the C-terminal protecting groups employed,^{21–24} i.e. during the alkaline saponification of methyl esters, the hydrogenolysis of benzyl esters, the acidolysis of *tert*-butyl esters, and the Pd(0)- or Rh(I)-catalyzed removal of allyl esters. If the construction of PhAc dipeptides is carried out by chemical activation of the PhAc amino acids, the



peptide	R	yield [%]	ref(s)
Gly-Phe	tBu	65	21, 22
Thr-Ala	tBu	75	21, 22
Ala-Ala	tBu	90	21, 22
Ser-Leu	tBu	95	21, 22
Asp(OH)-Phe	Me	87	20

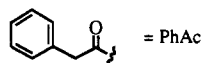


Figure 3.

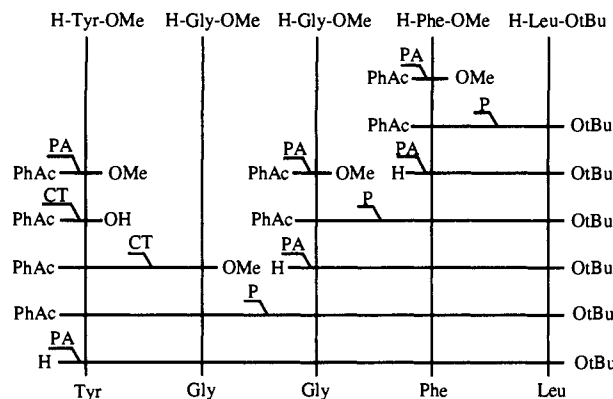
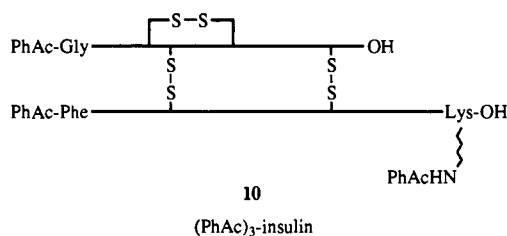
application of the non-urethane blocking group results in ca. 6% racemization.^{21,22} However, this disadvantage can be overcome by forming the peptide bonds enzymatically, e.g. with trypsin,³⁶ chymotrypsin,³⁶ or carboxypeptidase Y.^{31,38} For these condensation reactions and the subsequent enzymatic removal of the PhAc group, a continuous process was developed which has the potential to be amenable to a larger scale.³¹

The applicability of the penicillin acylase catalyzed deprotection for the construction of larger peptides has been demonstrated in the complete deprotection of the porcine insulin 10 carrying three PhAc groups,¹⁹ presumably at the N-terminal glycine of the A chain, the N-terminal phenylalanine of the B chain, and the side chain of the lysine in position 29 of the B chain (Figure 4). The enzymatic hydrolysis proceeded to completeness and the peptide backbone was not attacked. A further interesting example is given in a recent biocatalyzed synthesis of leucine enkephalin *tert*-butyl ester (11)³⁰ in which all critical steps are performed by enzymes, two of them through the agency of penicillin G acylase: (i) phenylacetates are introduced as N-terminal protecting groups of the amino acid esters by using penicillin G acylase, (ii) the elongation of the peptide chain is carried out with papain or α -chymotrypsin, (iii) the deprotection of the N-terminal amino group is achieved again by means of penicillin G acylase. These examples and also the application of this technique for aspartame synthesis,^{20,32,33} as well as the deprotection of glutathione derivatives²⁷ demonstrate that penicillin G acylase can advantageously be used for the N-terminal unmasking of peptides. In addition, the enzyme has been applied for the liberation of the side-chain functional groups of lysine³⁸ (Figure 5) and cysteine²⁵⁻²⁷ (Figure 9), as well as in β -lactam^{33,39-41} (Figures 6 and 7), nucleoside⁴³ (Figure 8), and carbohydrate chemistry^{22-24,104} (Figure 24).

The enzyme-labile N-protecting functions developed so far are simple acyl groups which embody the danger of racemization during chemical peptide syntheses. This problem can, in general, be overcome by the use of urethane blocking functions. Since biocatalysts are known³⁷ which cleave Z and Boc groups from amino acids, their application in peptide synthesis can be foreseen.

2.2. Protection of the Side-Chain Amino Group of Lysine

During chemical peptide synthesis and if trypsin is used for the construction of the peptide bonds or



PA: penicillin G acylase; CT: chymotrypsin; P: papain

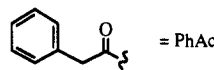


Figure 4.

N-terminal deprotection, the side-chain amino group of lysine generally has to be protected to prevent side reactions.⁶ This goal can be achieved enzymatically by applying the penicillin G acylase catalyzed removal of the PhAc group³⁸ (vide supra, Figure 4). Thus, the first application of the PhAc group in peptide chemistry consisted in a synthesis of 1-deamino-Lys⁸-vasopressin (12), during which the lysine side chain was masked as the phenylacetamide (Figure 5). After the peptide chain had been assembled and the disulfide bond was formed by oxidative cyclization, the PhAc group could be removed enzymatically in 74% yield without side reaction. A further interesting example which demonstrates that this technique can advantageously be applied to the synthesis of even larger peptides is found in the complete deprotection of tris(phenylacetamido)-porcine insulin (10)¹⁹ (vide supra, Figure 4). Since penicillin acylase is commercially available and devoid of peptidase activity, this method appears to be generally useful for the construction of lysine-containing oligopeptides.

In addition to the PhAc group, pyroglutamyl amides (Glp) were proposed as enzymatically removable blocking functions for the lysine side chain.¹⁵ Their removal was achieved with pyroglutamate aminopeptidase from calf liver. Thus, all N-protecting groups were cleaved from the protected RNase 1-10 fragment Glp-Lys(Glp)-Glu-Thr-Ala-Ala-Ala-Lys(Glp)-Phe-Glu-Arg-OH and from a model dipeptide. However the general usefulness of this method remains to be demonstrated.

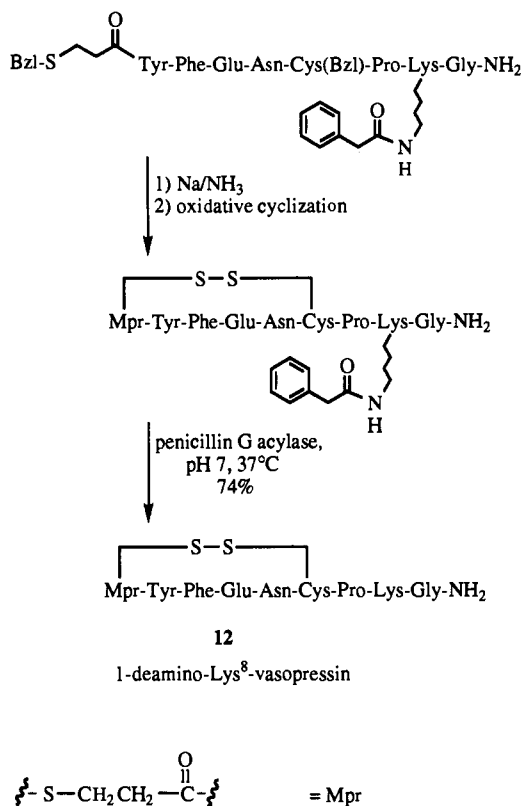


Figure 5.

2.3. Protection of Amino Groups in β -Lactam Chemistry

The enzymatic removal of acyl groups plays an important role in the industrial production of semisynthetic penicillins and cephalosporins. To this end, penicillin G and penicillin V (13) or the respective cephalosporins (14) are first deacylated by means of penicillin acylases^{39,40} (Figure 6). The 6-aminopenicillanic acid (15) and the 7-aminocephalosporanic acid (16) thus obtained are subsequently acylated by chemical or enzymatic methods to give the semisynthetic antibiotics 17 and 18, e.g. ampicillin and cephalixin.

The manufacturing of therapeutically important cephalosporins from penicillin G and V includes a chemical ring expansion of the thiazolidine ring to a dihydrothiazine. In the course of this sequence the amino group remains protected as phenylacetyl or phenoxyacetyl amide which is finally removed using penicillin G or V acylase. Of particular importance is the choice of a suitable protecting function for the COOH group. It must be stable during the ring expansion but removable without damaging the cephalosporin nucleus. As an alternative to chemical methods, the use of the (phenylacetoxy)methylene ester was suggested for this purpose.^{33,41} It is easily introduced into penicillin G and the corresponding sulfoxide 19 and is stable during the construction of the cephalosporin framework (Figure 7). Together with the phenylacetamide the ester can finally be removed in high yield from penicillin G, the sulfoxide 20, and the cephalosporins 21 by penicillin G acylase. The form-

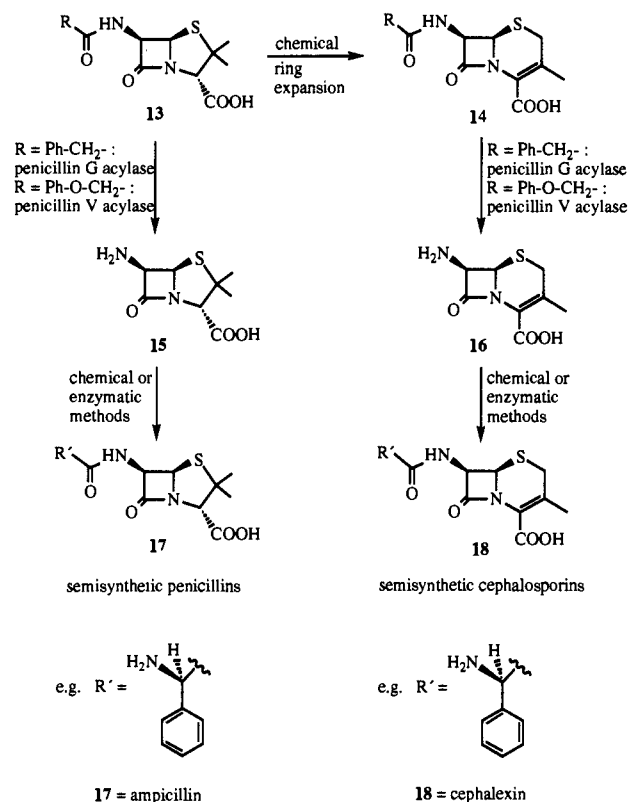


Figure 6.

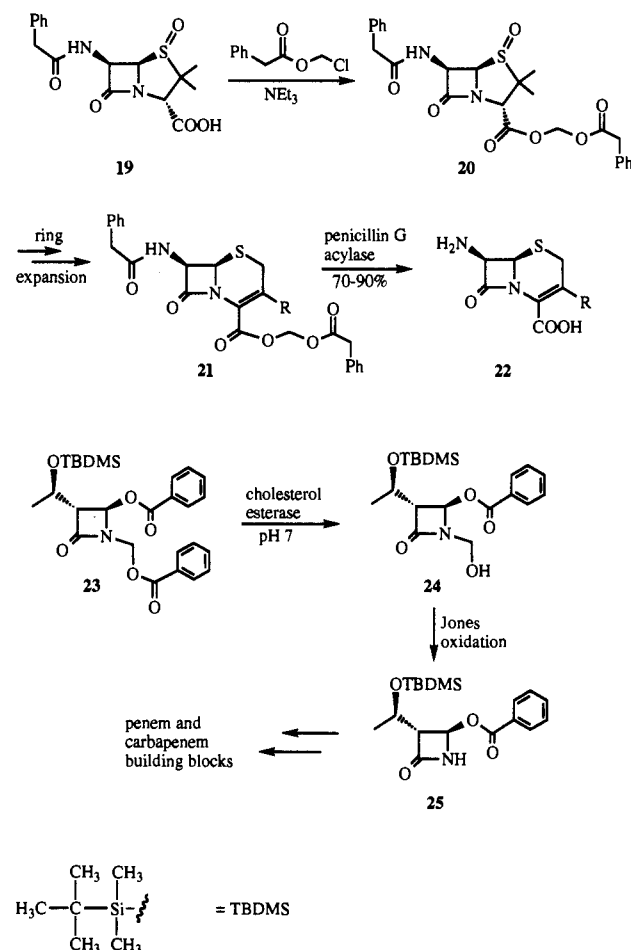
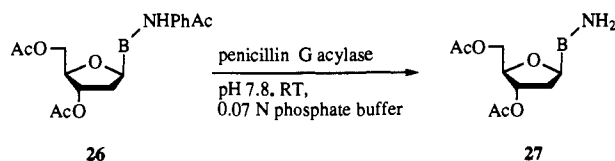


Figure 7.

aldehyde formed in the deprotection is not harmful to the enzyme.



		yield [%]	ref
		50	43
		69	43
		50	43

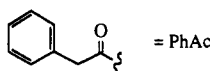


Figure 8.

In a new approach to well-known versatile β -lactam building blocks, an enzymatic deprotection of an acylated methylol amide was advantageously applied.⁴² Thus, the dibenzoate **23**, generated from L-threonine, was regioselectively saponified by cholesterol esterase at pH 7, giving rise to the aminal **24** (Figure 7). After Jones oxidation and subsequent loss of formaldehyde, the azetidinone **25** was obtained which can be transformed into various enantiomerically pure penem and carbenem building blocks.

2.4. Protection of Amino Groups of Nucleosides

The amino groups of the nucleobases adenine, guanine, and cytosine in general must be protected during oligonucleotide synthesis to prevent undesired side reactions. To this end, they usually are converted into amides which finally are hydrolyzed under fairly basic conditions. If the amino groups are, however, masked as phenylacetamides, the protecting functions can be cleaved off by employing penicillin G acylase⁴³ again (Figure 8). The enzyme, for instance, selectively liberates the amino groups of the deoxynucleosides **26** without attacking the acetates in the carbohydrate parts and without damage of the acid-labile N-glycosidic bonds.

3. Protection of Thiol Groups³⁻⁵

3.1. Protection of the Side-Chain Thiol Group of Cysteine

The liberation of the β -mercapto group of cysteine was achieved by means of the penicillin G acylase mediated hydrolysis of phenylacetamides.²⁵⁻²⁷ To this end, the SH group was masked with the phenylacetamidomethyl (PhAc) blocking function (Figure 9). After penicillin acylase-catalyzed hydrolysis of the

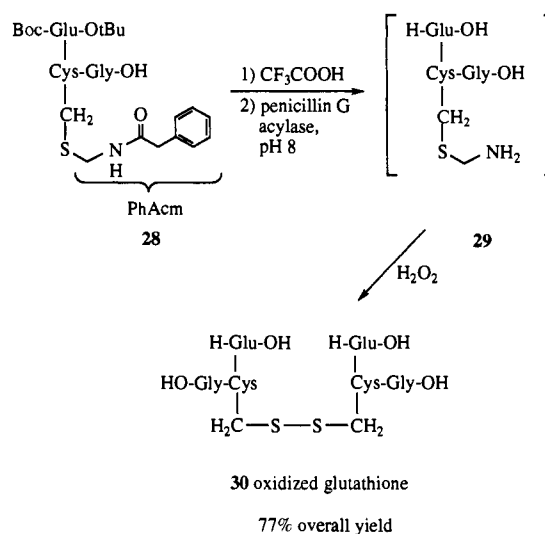


Figure 9.

amide incorporated in the acylated thioacetal (see, e.g. **28**), the labile S-aminomethyl compound (e.g. **29**) is formed and immediately liberates the desired thiol. This technique was for instance applied in a synthesis of glutathione which was isolated as the disulfide **30**. In a related glutathione synthesis the method was used for the simultaneous liberation of the SH- and the N-terminal amino function of glutamine.^{26,27}

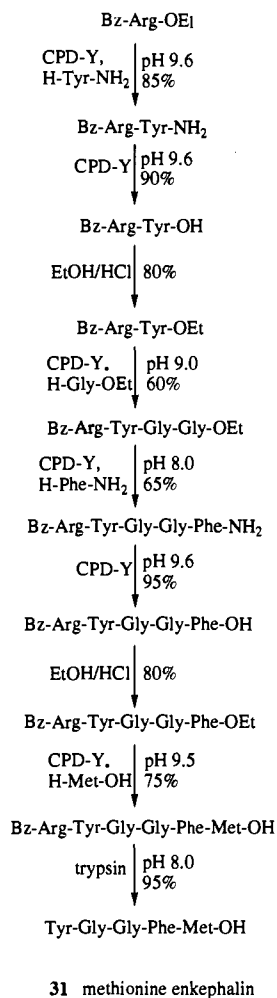
4. Protection of Carboxy Groups³⁻⁵

4.1. C-Terminal Protection of Peptides

As in the enzymatic liberation of the N-terminus of peptides, initial attempts to achieve an enzyme-catalyzed deprotection of the corresponding carboxy groups concentrated on the use of the endopeptidases chymotrypsin,⁴⁴⁻⁴⁶ trypsin,^{45,47,48} and thermolysin,⁴⁹ a protease obtained from *Bacillus thermoproteolyticus* which hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues (e.g. leucine, isoleucine, valine, phenylalanine). This latter biocatalyst served for the cleavage of the "supporting" tripeptide ester H-Leu-Gly-Gly-OEt from a protected undecapeptide (pH 7, room temperature). The octapeptide thereby obtained was composed exclusively of hydrophilic amino acids. Due to the broad substrate specificity of thermolysin and the resulting possibility of unspecific peptide hydrolysis this method cannot be regarded as being generally applicable.

The exploitation of the esterase activities of chymotrypsin and trypsin opened routes to the hydrolysis of several peptide methyl, ethyl, and *tert*-butyl esters at pH 6.4-8 and room temperature.^{44,45} The transformation is not only successful with peptides carrying the respective enzyme specific amino acids at the C-terminus, but in several cases also different amino acids were tolerated at this position. However, severe drawbacks of this methodology are that numerous peptides are poor substrates or not accepted at all. Moreover, a competitive cleavage of the peptide bonds occurs if the peptides contain trypsin- or chymotrypsin-labile sequences. Therefore, these proteases appear to be not generally useful for a safe C-terminal deprotection as well.

The disadvantages displayed by the endopeptidases can be overcome by using carboxypeptidase Y from



CPD-Y : carboxypeptidase Y

Figure 10.

bakers' yeast.^{17,50,51} This serine-exopeptidase also has esterase activity and is characterized by quite different pH optima for the peptidase and the esterase activity (pH >8.5). Even in the presence of various organic cosolvents the enzyme selectively removes the carboxy protecting groups from a variety of differently protected di- and oligopeptide methyl and ethyl esters^{17,51} without attacking the peptide bonds. An additional attractive feature is, that its esterase activity is restricted to α -esters, consequently β - and γ -esters of aspartic and glutamic acid, respectively, are not attacked. Carboxypeptidase Y (CPD-Y) was advantageously used for the stepwise elongation of the peptide chain in aqueous solution employing a solubilizing polymeric support as N-terminal blocking group.⁵⁰ The peptides were grown on carboxymethyl-poly(ethylene glycol)-glycylmethionine as a handle which on the one hand promotes solubility in aqueous media and on the other hand allows the release of the synthesized peptides by CNBr-mediated cleavage of the methionine amide to be carried out. The peptide chain is elongated in the C-terminal direction by successive couplings of amino acid ethyl esters to the free carboxy groups obtained by hydrolysis of the respectively peptide ethyl esters with carboxypeptidase Y at pH 8.5 and room temperature. By using this procedure, after cleavage with cyanogen bromide chemically and optically pure H-Leu-Phe-Leu-OEt was obtained in 64% overall yield. In a different

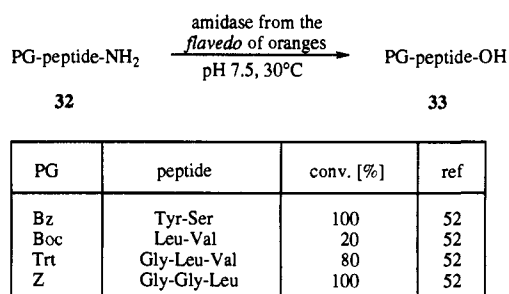


Figure 11.

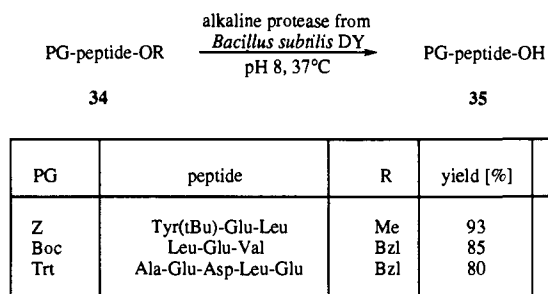


Figure 12.

remarkable synthesis which did not include the use of a polymeric N-protecting group, Met-enkephalin (31) was built up employing carboxypeptidase Y for C-terminal deprotection as well as for the formation of the peptide bonds¹⁷ (Figure 10).

In this synthetic scheme peptide ethyl esters served as acyl components for the condensation reactions. To avoid undesired oligomerizations as side reactions, amino acid amides proved to be the nucleophiles of choice. The resulting peptide amides then were saponified at pH 9.5 using again the enzyme. The carboxylic acids thereby formed subsequently had to be reesterified before the next coupling step could be carried out. *N*-Benzoylarginine was chosen as a solubilizing N-terminal protecting group which could be removed in the last step with trypsin (vide supra, Figure 1⁹⁻¹⁵). The additional opportunity to selectively hydrolyze C-terminal peptide amides with carboxypeptidase Y is of particular interest if, as is demonstrated in the above-mentioned example, enzymatic methods are applied for the formation of the peptide bonds. For this purpose a peptide amidase from the flavedo of oranges shows very promising properties.^{52,53} The enzyme is equipped with a broad substrate specificity and accepts Boc-, Trt-, Z-, and Bz-protected and N-terminally unprotected peptide amides (Figure 11).

The C-terminal amides 32 are saponified by the biocatalyst in high yields at pH 7.5 and 30 °C without affecting the N-terminal blocking groups or the peptide bonds.

A further possibility for the enzymatic removal of C-terminal blocking groups is opened up by the application of enzymes which generally display a high esterase/protease ratio. Such a biocatalyst is the alkaline protease from *Bacillus subtilis* DY which shows similarities to subtilisin Carlsberg. For this enzyme the ratio of esterase to protease activity is >10⁵. It selectively removes methyl, ethyl, and benzyl esters from a variety of Trt-, Z-, and Boc-protected di- and tripeptides and a pentapeptide at pH 8 and 37 °C⁵⁴ (for representative examples see Figure 12).

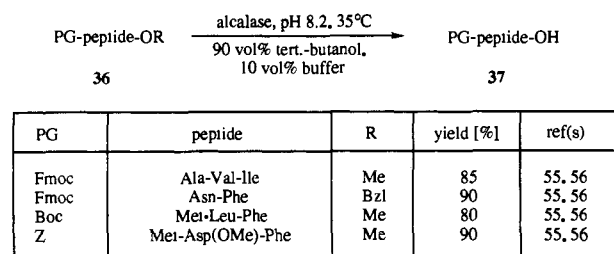


Figure 13.

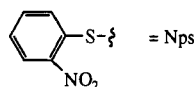
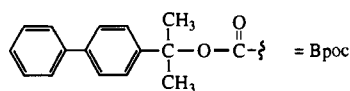
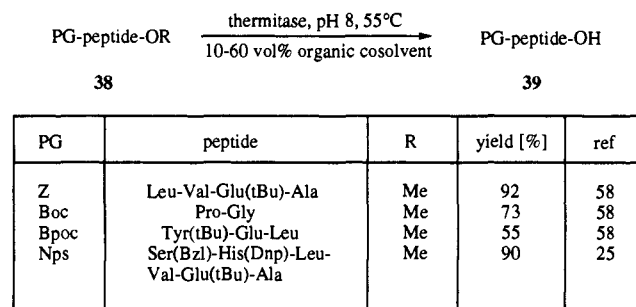
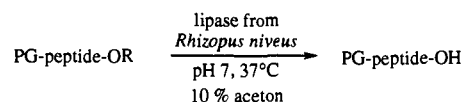


Figure 14.

The N-terminal urethanes and the peptide linkages are left intact. A further protease which fulfills the requirements for a successful application in peptide chemistry is alcalase, a serine endopeptidase from *Bacillus licheniformis* whose major component is subtilisin A (subtilisin Carlsberg).⁵⁵⁻⁵⁷ It can advantageously be employed to selectively saponify peptide methyl and benzyl esters (for representative examples see Figure 13). In a solvent system consisting of 90% *tert*-butyl alcohol and 10% buffer (pH 8.2) even highly hydrophobic and in aqueous solution insoluble Fmoc peptides were accepted as substrates and deprotected at the C-terminus without any disturbing side reaction. A selective classical alkaline saponification of methyl esters would be impossible due to the base sensitivity of the Fmoc group.

A very promising and unusually stable biocatalyst is thermitase, a thermostable extracellular serine protease from the thermophilic microorganism *Thermoactinomyces vulgaris* whose esterase/protease ratio amounts to >1000:1. The enzyme shows a broad amino acid side chain tolerance and cleaves methyl, ethyl, benzyl, ethoxybenzyl, and *tert*-butyl esters from a variety of Nps-, Boc-, Bpoc-, and Z-protected di- and oligopeptides in high yields at pH 8 and 35–55 °C^{25,26,58-60} (for representative examples see Figure 14). In addition it is specific for the α -carboxy groups of Asp and Glu. To enhance the solubility of the substrates up to 50 vol % of organic cosolvents like DMF and DMSO may be added which also serve to reduce the remaining peptidase activity to a negligible amount.^{26,60}

In the discussion of the protease-catalyzed cleavage of the N-terminal protecting groups it was already

40 R = (CH₂)₆CH₃ = Hep41 R = (CH₂)₂Br = EtBr42 R = (CH₂)₂-N O = MoEt

PG	peptide	R	yield [%]	ref(s)
Boc	Ser-Thr	Hep	95	3, 4, 61, 62
Z	Thr-Ala	Hep	85	3, 4, 24, 61, 62
Aloc	Met-Gly	Hep	90	3, 4, 24, 61, 62
Z	Ser-Phe	EtBr	84	3, 4
Boc	Val-Ala	EtBr	95	3, 4, 24
Boc	Val-Phe	MoEt	91	64

Figure 15.

pointed out that the use of biocatalysts belonging to this class of enzymes in general, i.e. also for the C-terminal deblocking, may lead to an undesired hydrolysis of peptide bonds. In particular, this has to be expected if the respective ester or amide to be hydrolyzed turns out to be a poor substrate which is only attacked slowly, an experience not uncommon if unnatural substrates are subjected to enzyme-mediated transformations. This undesired possibility would, however, be overcome if enzymes were used which are not able to split amides at all. This principle has been realized in the development of the heptyl (Hep)^{3,4,23,24,61,62} and the 2-bromoethyl (EtBr) esters^{3,4,23,24,63} as carboxy protecting groups for peptide synthesis which can be enzymatically removed by means of lipases (Figure 15).

The amino acid heptyl esters, required for the stepwise elongation of the peptide chain, are conveniently synthesized in high yields by azeotropic esterification. The Hep esters prove to be chemically stable during the removal of the N-terminal Z, Boc, and the Aloc group from the corresponding dipeptides 40, i.e. they are not affected by hydrogenation and by treatment with HCl/ether and in the presence of Pd(0) catalysts and C-nucleophiles. The selective removal of the heptyl esters is achieved by a lipase-catalyzed hydrolysis. From several enzymes investigated, a biocatalyst isolated from the fungus *Rhizopus niveus* was superior to the others with respect to substrate tolerance and reaction velocity. The enzyme accepts a variety of Boc-, Z-, and Aloc-protected dipeptide heptyl esters as substrates and hydrolyzes the ester functions in high yields at pH 7 and 37 °C without damaging the urethane protecting groups and the amide bonds (for representative examples see Figure 15).^{61,62} Z- and Boc-dipeptide 2-bromoethyl esters 41 are attacked, too, with comparable or in some cases even higher velocity. In the presence of either one of the enzyme-labile protecting groups the N- and C-terminal amino acid can be varied in a wide range. With increasing steric bulk and lipophilicity of the amino acids, especially the C-terminal one, the velocity of the enzymatic reactions decreases. Dipeptides containing two sterically demanding hydrophobic amino acids, in particular carrying phenylalanine at the C-terminus are only poor substrates for the enzyme or are not attacked at all. If the C-terminal amino acid is proline, the enzymatic reaction does not take place either. The problem that heptyl esters of

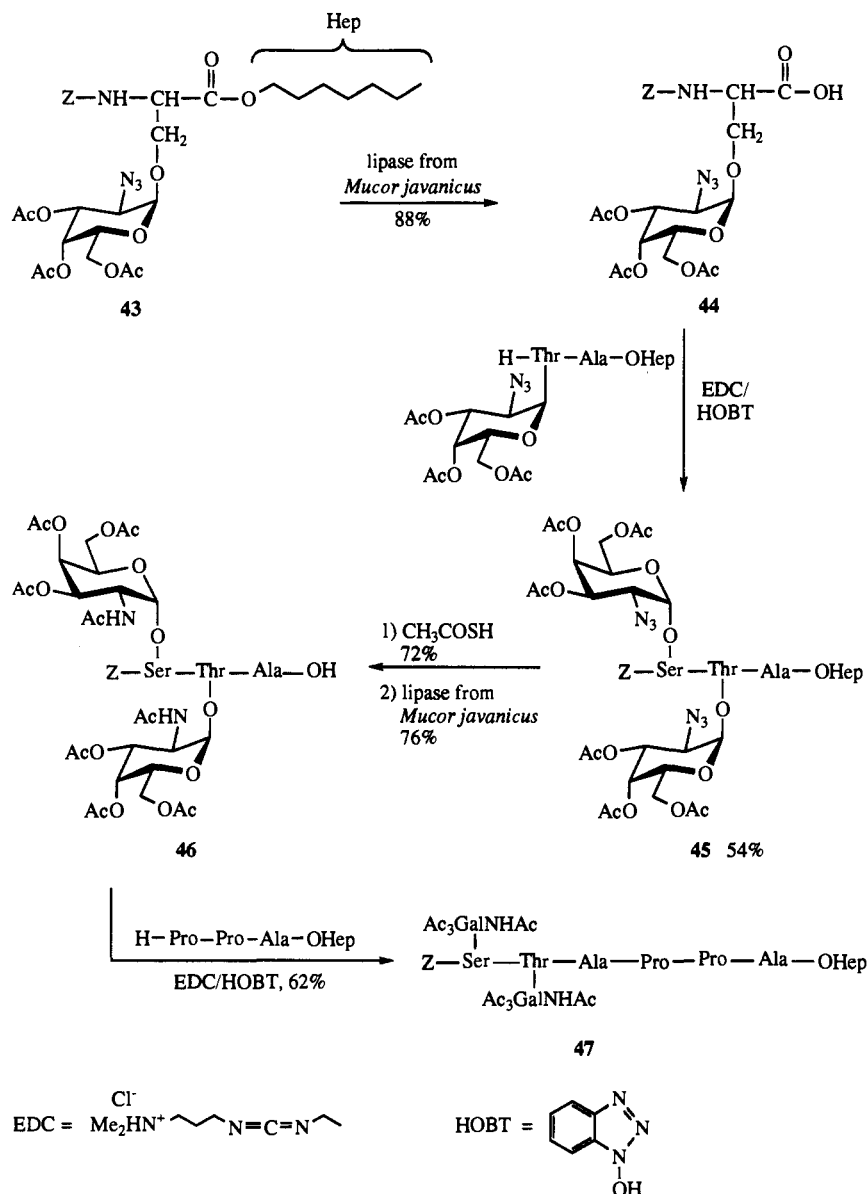


Figure 16.

hydrophobic peptides frequently are not hydrolyzed by lipases, in many cases can be overcome by the use of hydrophilic esters which guarantee that the substrate becomes better accessible to the enzyme. Thus, recently it was shown that the markedly hydrophilic 2-(*N*-morpholino)ethyl esters (MoEt), e.g. **42**, of protected peptides also were selectively and in good yields cleaved by means of lipase from *R. niveus*⁶⁴ (Figure 15). The respective dipeptide heptyl ester was not attacked by the enzyme at all.^{61,62}

The use of lipases for the removal of protecting groups from peptides in addition to the absence of protease activity has several advantages. Various enzymes belonging to this class and stemming from different natural sources (including mammals, bacteria, fungi, and thermophilic organisms) are commercially available and rather inexpensive. This variety gives the opportunity to replace a chosen biocatalyst by a better one if a particular substrate is only attacked slowly (vide infra, Figure 16). The lipases are not specific for L-amino acids but also tolerate the presence of the D-enantiomer.⁶⁵ A noticeable feature is that, in contrast to proteases and esterases, they operate at the interface

between water and organic solvents.⁶⁶ This is particularly important if longer peptides, being composed of hydrophobic amino acids and/or carrying side-chain protecting groups, and which do not well dissolve in the aqueous systems, have to be constructed.

The full capacity of the lipase mediated technique for C-terminal deprotection was demonstrated in the synthesis of complex *O*-glycopeptides which are sensitive to both acids and bases.⁶⁷ To this end, e.g. the serine glycoside heptyl ester **43** was selectively deprotected at the C-terminus by lipase from the fungus *Mucor javanicus* (Figure 16).

The carboxylic acid **44** liberated thereby was then condensed with an N-terminally deprotected glycopeptide to yield the diglycotriptide **45**. After conversion of its azido groups into acetamides the enzyme-mediated deprotection could be carried out again, delivering the glycotriptide carboxylic acid **46** in high yield. This compound was finally condensed with a tripeptide to give the complex diglycohexapeptide **47** which carries the characteristic linkage region of a tumor-associated glycoprotein antigen found on the surface of human breast cancer cells. In the course

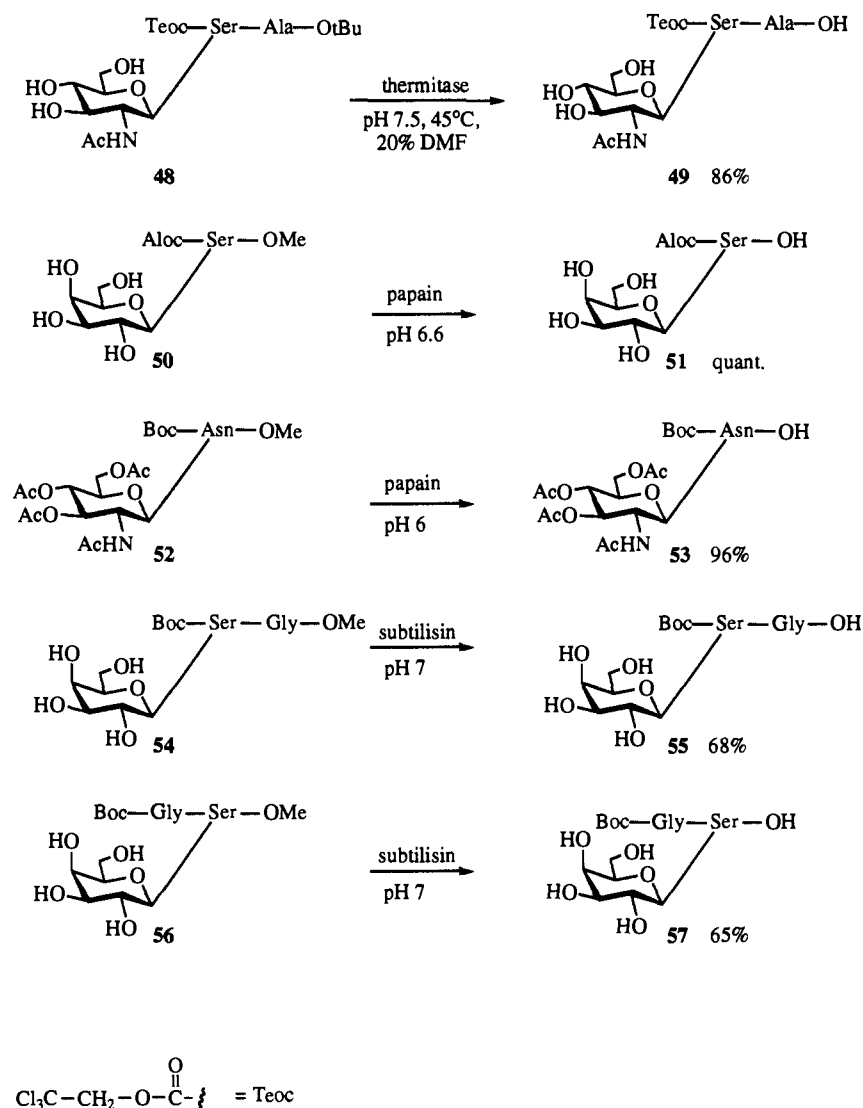


Figure 17.

of these enzymatic transformations the N-terminal urethanes, the peptide bonds, the acid- and base-labile glycosidic linkages, and the acetyl protecting groups, being sensitive to bases, were not attacked. In these cases, lipase N which was the enzyme of choice for simple peptides attacked the substrates only slowly, so that a different biocatalyst had to be used. This demonstrates the above-mentioned advantage of being able to apply several catalytic proteins of comparable activity but different substrate specificity for the solution of a given synthetic problem. In addition to the heptyl esters, the 2-(*N*-morpholino)ethyl (MoEt) protecting group (vide supra) can also be removed from *O*-glycopeptides by means of lipase N.⁶⁴

The viability and the wide applicability of the principle to use enzymes for the removal of individual protecting groups from complex multifunctional compounds like glycopeptides is furthermore proven by the finding that also proteases can be used for this purpose. Thus, by means of thermitase catalysis the C-terminal *tert*-butyl ester was removed from the glycopeptide 48^{26,68} (Figure 17). From the serine glycoside 50^{69,70} and from the asparagine conjugate 52⁷¹ the methyl esters could be cleaved off without a disturbing side reaction by using papain as biocatalyst (Figure 17). Similarly, the liberation of the C-terminal carboxy group of the

glycosylated dipeptides 54 and 56 was achieved by means of subtilisin-catalyzed hydrolysis.⁷² However, in these cases papain could not be used since this protease preferably cleaved the peptide bonds. This example highlights again the danger associated with the use of a protease for the removal of protecting groups from peptides.

A regularly arising problem in the enzymatic deprotection is the poor solubility of the fully blocked peptides in the required aqueous media, resulting in a limited accessibility of the substrates to the enzymes. To overcome this difficulty, in many cases solubilizing organic cosolvents can be added; however, as a guideline for future developments it is to be expected that a more general and viable approach should consist in the development of solubilizing protecting groups. Similar findings were already recorded in the enzyme-mediated formation of peptide bonds.⁷³

4.2. Protection of the Side-Chain Groups of Glutamic and Aspartic Acid

The stepwise removal of arginine methyl ester by proteases has been investigated as a possibility for the enzymatic deprotection of the side chain carboxylate groups of the amino dicarboxylic acids aspartic acid

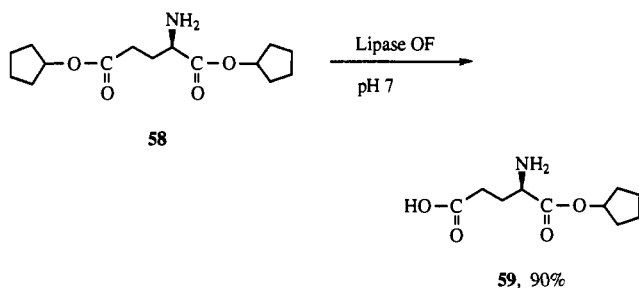
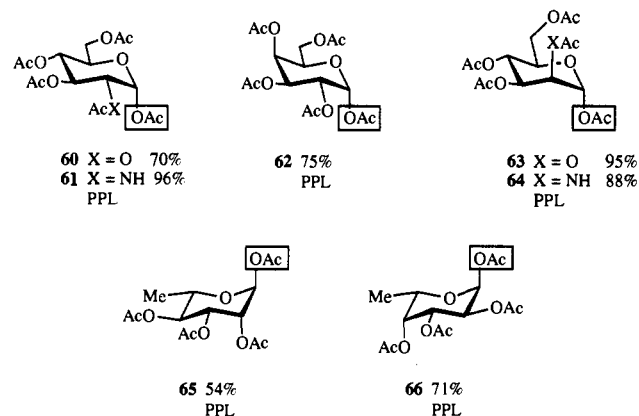


Figure 18.



PPL = lipase from porcine pancreas

Figure 19.

(Asp) and glutamic acid (Glu). To this end, Z-Asp(ArgOMe)-NH₂ and Z-Glu(ArgOMe)-NH₂ were converted to Z-Asp(OH)-NH₂ and Z-Glu(OH)-NH₂ by subsequent treatment with trypsin, which hydrolyzes the arginine methyl esters and with porcine pancreatic carboxypeptidase B which splits off the arginines.⁷⁴ Since the second step is slow and requires high concentrations of the carboxypeptidase, this method most probably cannot be routinely applied in peptide synthesis because it introduces too much danger of competitive side reactions. However, enzymatic transformations have proven to be useful for the synthesis of selectively functionalized aspartic and glutamic acid derivatives. For instance, alcalase selectively hydrolyzes the α -benzyl esters of H-Asp(Bzl)-OBzl and H-Glu(Bzl)-OBzl in 82% and 85% yield, respectively, on a decagram scale.⁷⁵ Similarly, aspartyl- and glutamylpeptides can be deprotected selectively at the C-terminus by this enzyme; however, in these cases an undesired attack on the peptide bonds may occur.⁷⁶ In addition, Z-Asp(OAll)-OAll is converted to Z-Asp(OAll)-OH in quantitative yield by papain.⁷⁷ Also a lipase from *Candida cylindracea* is able to differentiate between the two carboxylic acid groups of glutamic acid. From the respective dicyclopentyl ester 58 it preferably (ratio 20:1) removes the γ -ester in 90% yield⁷⁸ (Figure 18). In addition, the enzyme thermitase and the alkaline protease from *B. subtilis* (vide supra) hold a great potential for the selective manipulation of dicarboxylic amino acids.

The examples given in sections 2.1 to 4.2 demonstrate that the selective deprotection of peptides can be achieved advantageously by making use of enzymatic reactions. In the light of the increasing number of available biocatalysts (including catalytic antibodies)

it appears that in the near future a host of new and superior enzymatically removable blocking groups for the construction of peptides will be developed. However, these techniques will certainly not be used for the construction of simple small peptides in the laboratory. Most probably they will be applied for the synthesis of sensitive polyfunctional compounds and long oligopeptides whose construction is cumbersome by standard chemical methods. In addition, they offer significant advantages if a technical process for the synthesis of a given peptide has to be developed. Finally, together with the recently developed methods for the biocatalyzed formation of peptide bonds,⁷⁹ enzymatic protecting group techniques could prove to be the tools of choice for the construction of peptides in aqueous solution which one has tried to develop to practicability for several decades.^{80,81}

5. Protection of Hydroxy Groups^{3,4,82,83}

Mono- and oligosaccharides, alkyl- and arylglycosides, and various other glycoconjugates generally embody a multitude of hydroxyl groups of comparable chemical reactivity. In addition, the synthesis of oligonucleotides and nucleosides, β -lactams, alkaloids, steroids, and peptides often requires the selective protection of one or more alcoholic functions. Consequently, for the directed construction of polyhydroxy compounds these functional groups have to be manipulated selectively, in general making cumbersome protection and deprotection steps necessary. Although numerous chemical techniques are available to mask or to liberate hydroxyl groups,^{1,2} the development of enzymatic methods for this purpose has been progressing steadily and appears to complement the arsenal of classical tools.

5.1. Protection of Carbohydrates

The selective protection and deprotection of carbohydrates can be achieved with various classical chemical techniques.^{1,2,84,85} In addition, however, due to the synthetic challenge posed by the multifunctional carbohydrates, enzymatic techniques for the introduction of blocking groups into sugars and/or their subsequent removal offer further, different opportunities.

5.1.1. Monosaccharides

5.1.1.1. Pyranoses. Initial attempts to apply lipases for the enzymatic removal of acyl groups from glucose pentaacetate resulted in only low levels of selectivity.^{86,87} However, later on, lipase from porcine pancreas (PPL) was found to hydrolyze exclusively the anomeric acetate from the peracetylated pyranoses 60–66 in 54–96% yield⁸⁸ (Figure 19).

On the other hand, if the anomeric center is derivatized as a methyl glycoside, the regioselective enzymatic liberation of the 6-OH group becomes feasible.^{88,89} Thus, from methyl α - and β -D-glucose tetrapentanoate 67 and the corresponding tetraoctanoate, lipase from *C. cylindracea* (CCL) removes only the primary ester group in yields of ca. 75%. Similarly, the α -D-galactoside 68, the mannoside 69, and the 2-acetamido-2-deoxymannoside 70 were converted to the 6-deprotected pyranosides in 29–50% yield (Figure 20), but the 2-acetamido-2-deoxyglucoside was only a poor substrate. In the latter cases the regioselectivity was less pronounced

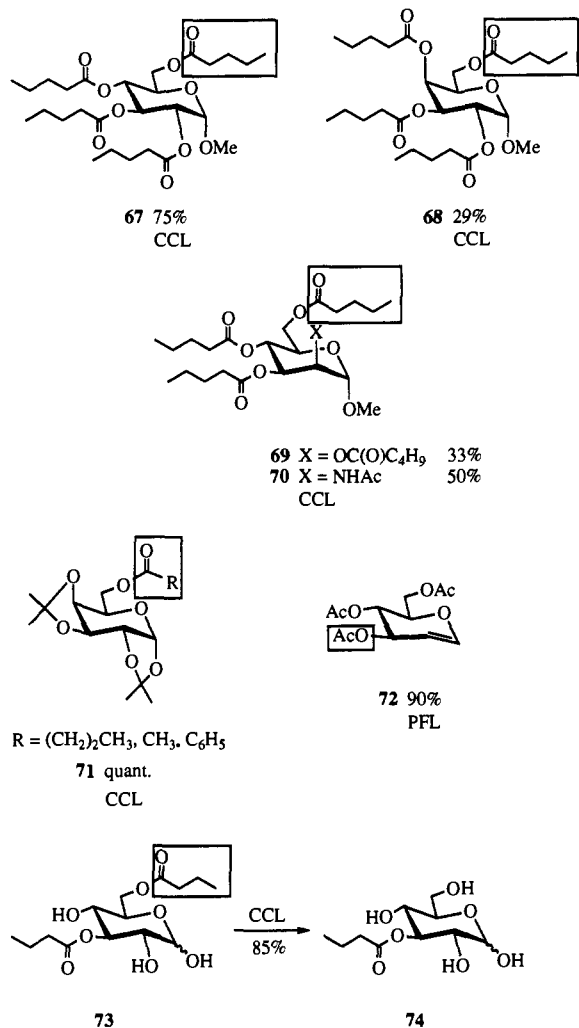
CCL = lipase from *Candida cylindracea*PFL = lipase from *Pseudomonas fluorescens*

Figure 20.

and the 4,6-diacylated derivatives were formed in ca. 20% yield, too. In addition to this class of compounds, lipases also accept pyranosides carrying several different functional groups (e.g. acetals),⁹⁰ like 71, and enol ethers,^{91,92} like 72 (Figure 20) and, in particular, 1,6-anhydropyranoses (vide infra, Figure 21) as substrates. In all cases the reaction conditions are so mild that the acid sensitive structures of these compounds remain unaffected. Particularly remarkable is the regioselectivity displayed by lipase from *Pseudomonas fluorescens* (PFL) in the deprotection of the glycol 72.^{91,92} The biocatalyst exclusively attacks the 3-acetate and leaves the primary ester intact. The enzymatic deprotection strategy can also be used to synthesize carbohydrates carrying a single acyl group in selected positions. Thus, 3,6-dibutyrylglucose 73 (prepared by enzymatic acylation of glucose; vide infra, Figure 26) was converted to the 3-butyrated 74 by lipase mediated hydrolysis of the 6-ester.⁹³

1,6-Anhydropyranoses serve as convenient starting materials for various synthetic purposes in carbohydrate chemistry. Therefore, the directed manipulation of their hydroxyl groups is of particular interest. Each of the three OH groups in 1,6-anhydroglucopyranose can be liberated selectively making use of enzymatic

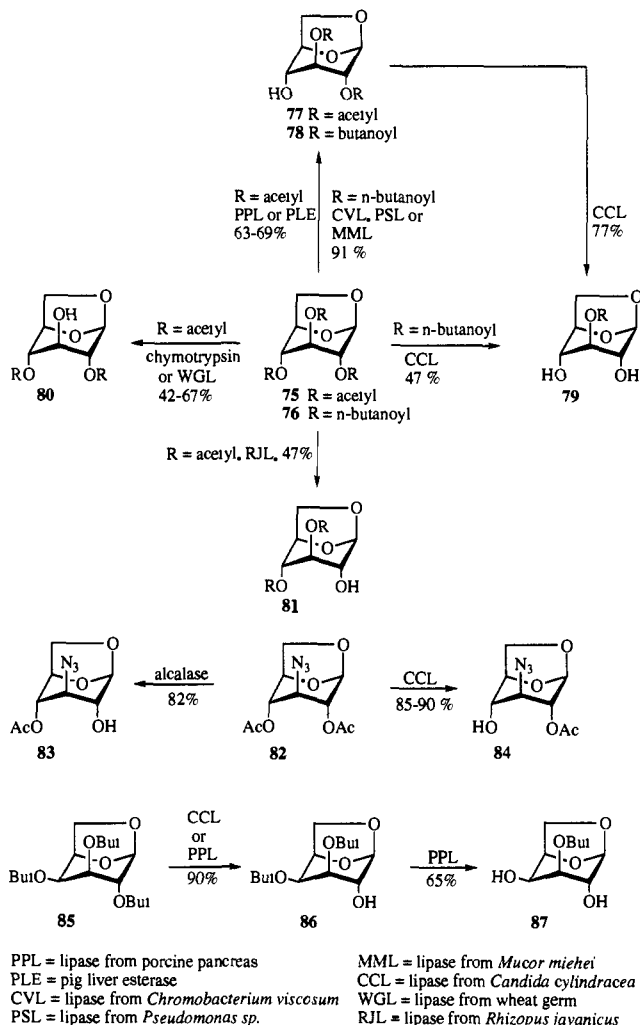


Figure 21.

reactions⁹⁴⁻⁹⁷ (Figure 21). Thus, from the triacetate 75 the 4-protecting group was split off using lipase from porcine pancreas (PPL)⁹⁵ or pig liver esterase (PLE).^{95,96} The acetate in the 3-position could be attacked preferentially using chymotrypsin⁹⁵ or lipase from wheat germ (WGL),⁹⁶ and the 3,4-diacetate 81 was obtained by hydrolysis with lipase from *R. javanicus* (RJL).⁹⁶ In each case, however, other derivatives were formed as undesired byproducts. High yields could be obtained by employing the tri-*n*-butanoate 76. It was converted to the 2,3-dibutanoate 78 in 91% yield by means of several lipases, but the enzyme from *C. cylindracea* (CCL) removed two acyl groups from 76 successively to yield the monobutyrated 79.⁹⁴ Similarly, the 3-azido-1,6-anhydropyranose 82 is regioselectively deacetylated at O-2 and O-4 by means of alcalase and lipase OF from *C. cylindracea*, respectively.⁹⁸ Of particular importance is the stereochemistry at C-4 of the bicyclic substrates. If the alcohol at this position is equatorial, as for instance in the corresponding 1,6-anhydrogalactopyranose 85, several enzymes act only in a random fashion or not at all.⁹⁴ However, lipase from *C. cylindracea* (CCL) and lipase from porcine pancreas (PPL) can be used to selectively hydrolyze the 2-butanoate in high yield⁹⁷ (Figure 21).

On prolonged reaction PPL also attacks the equatorial 4-ester, thus making the 3-protected 1,6-anhydro sugar 87 available in 65% yield.⁹⁷ Particularly remarkable

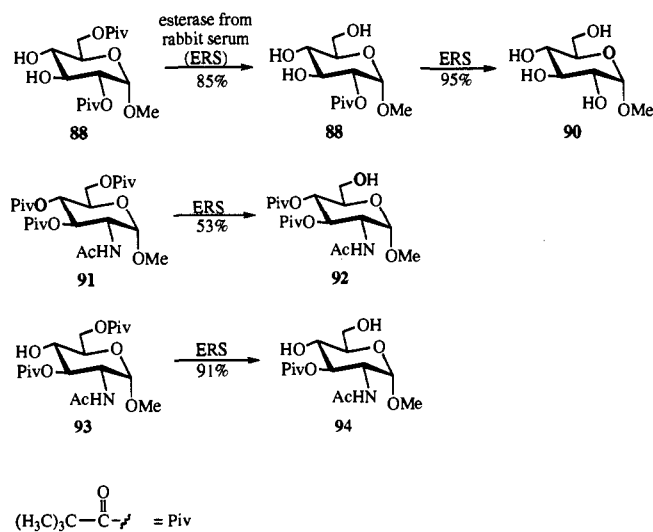


Figure 22.

is, that the enzyme displayed a markedly improved performance if it was immobilized on agarose.

The above-mentioned investigations unraveled that the lipase-mediated hydrolysis proceeds with higher velocity and, in many cases with better selectivity, if butanoates or pentanoates are employed as substrates instead of acetates. However, by no means is the use of enzymatic deacylations restricted to unbranched alkanooates. An illustrative and impressive example is found in the hydrolysis of generally base-stable carbohydrate pivaloates using an esterase from rabbit serum (ERS).⁹⁹⁻¹⁰² For instance, the biocatalyst selectively splits off the 6-pivaloyl groups from the pyranoses 88, 91, and 93 (Figure 22).

On prolonged incubation the complete removal of pivaloyl groups from carbohydrates is also possible. Of particular significance is that the enzyme does not have to be purified, but that crude serum preparations are sufficient for the preparative purposes. A further enzyme which allows the chemo- and regioselective unmasking of different carbohydrate derivatives to be carried out is acetyl esterase from the flavedo of oranges, a biocatalyst which hydrolyzes preferably acetic acid esters.^{24,43,103} The enzyme removes the acetate from the galactose 95 in high yield without any detectable side reaction (Figure 23). In addition, it can be applied for the synthesis of selectively deacetylated pyranoses. Thus, from pentaacetylglucose 97 the 2,3,4,6-tetraacetate 98 is obtained by means of the regioselective saponification of the 1-acetate. If the hydrolysis is allowed to proceed further, also the 2-acetate is cleaved regioselectively and the 3,4,6-triacetate 99 becomes available in 40% yield. If tri-*O*-acetyl glucal 100 is subjected to the enzymatic hydrolysis, at 66% conversion the 6-acetate 101 is the main product.^{43,103}

By introducing acyl groups which are specifically recognized by certain enzymes into carbohydrates, not only the regioselectivity but also the chemoselectivity of the biocatalysts can be exploited. This can, for instance, be achieved by the selective saponification of phenylacetates catalyzed by penicillin G acylase.²²⁻²⁴ The enzyme liberates the 2-OH group of the acylated glucose derivative 102 without affecting the acetic acid esters. In this case, moreover, an ester of a secondary hydroxyl function is chemoselectively hydrolyzed in

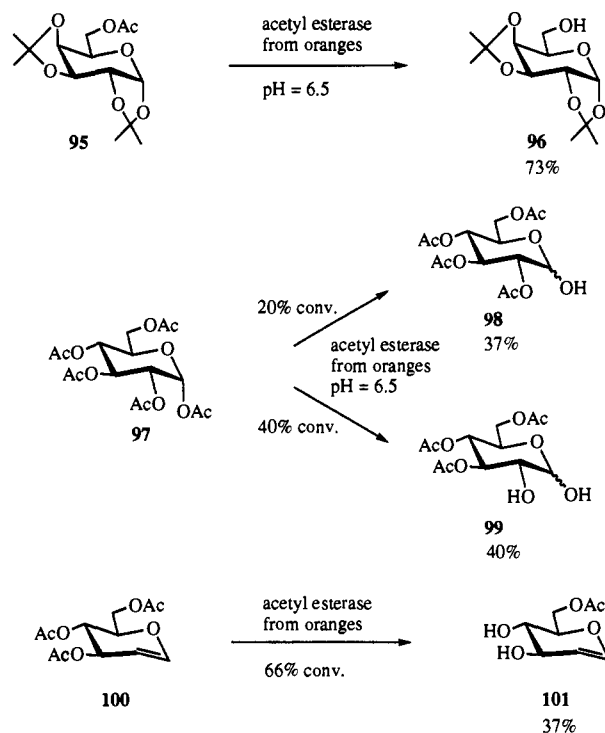


Figure 23.

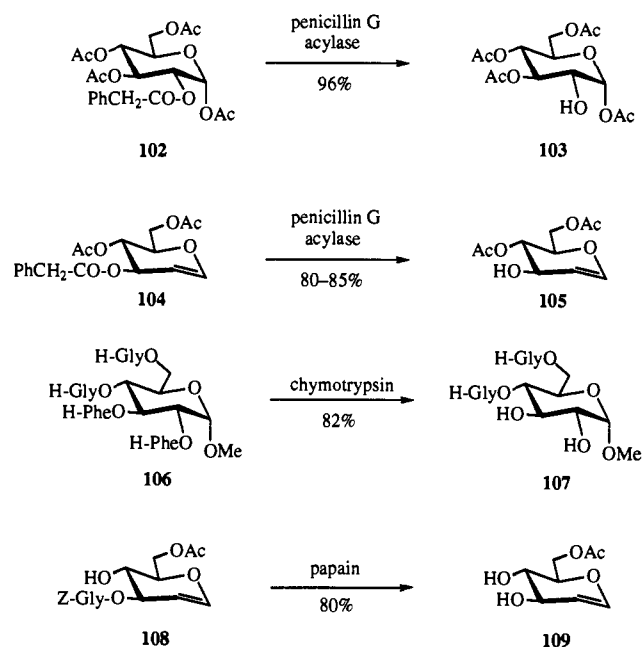


Figure 24.

the presence of the chemically more reactive acetates at the 6-position and at the anomeric center. This approach was also adopted for the enzymatic deprotection of the glucal 104. Thus, its 3-OH group was liberated without cleaving the present acetates.¹⁰⁴ Similarly, the catalytic abilities of proteases can be exploited. For instance, in accordance with its amino acid specificity, chymotrypsin attacks phenylalanine esters carrying pyranoses as the alcohol parts. Thus, from the protected methyl glycoside 106 it only hydrolyzed the phenylalanine esters and left the glycine residues unattacked¹⁰⁵ (Figure 24). The selectively protected diglycyl hexose 107 was formed in 82% yield. Similarly, papain found an advantageous application in the chemistry of glucals, e.g. 108¹⁰⁴ (Figure 24).

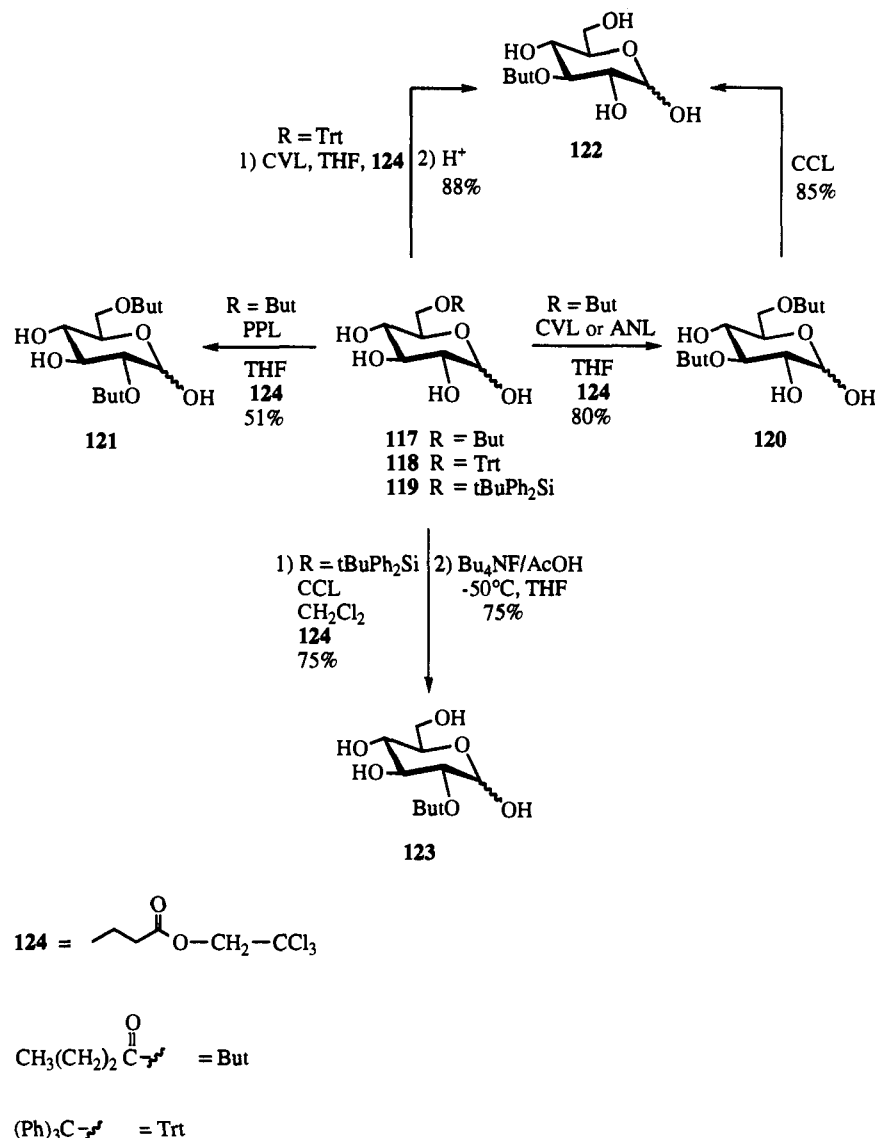


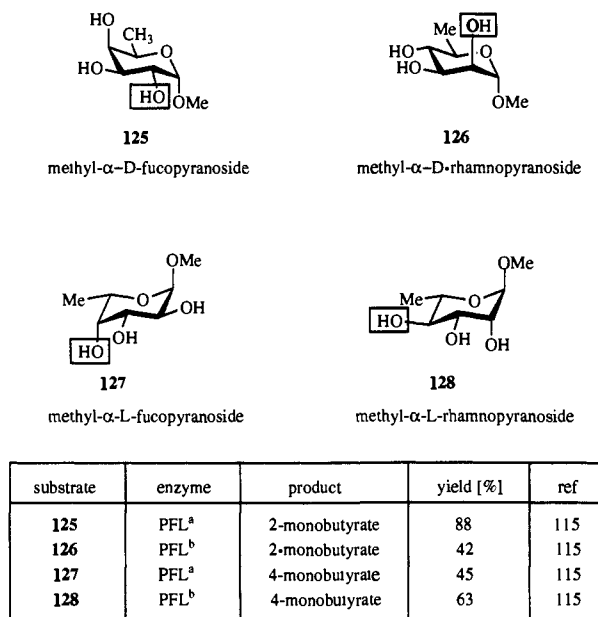
Figure 26.

The naturally occurring L enantiomers **127** and **128** of these 6-deoxy sugars, however, were esterified preferably at the 4-hydroxyl groups. These results contrast with chemical derivatizations, since the 4-hydroxyl groups of the 6-deoxy-L-carbohydrates have only very little reactivity toward chemical acylating reagents. In addition, methyl-L-fucoside can be converted to the 3-butyrate with lipase from *C. cylindracea*. The introduction of an acyl substituent into the 6-positions of the D-fucoside **125** and the L-rhamnoside **128** does not influence the regioselectivity of the enzymatic acylation.¹¹⁶ If the respective 6-O-butyrylated D-galactoside (which corresponds to **125**) is acylated by lipase from *P. fluorescens* or porcine pancreas, again the 2-position is derivatized preferably (92–95%). If the 6-O-butyric acid ester of the L-mannoside (which corresponds to **128**) is subjected to the action of these enzymes, the 4-hydroxyl group is esterified with 90% selectivity.¹¹⁶

Enzymatic acyl transfer reactions also turned out to be viable methods for the complete differentiation of the hydroxyl groups of glycals.^{91,104} Thus, the 6-OH function of D-galactal **129** is regioselectively acetylated or benzoylated using lipases from *Candida* species (Figure 28). Also subtilisin can be employed in the

acetylation of glucals (such as **129**).¹¹⁷ Employing a lipase from *P. fluorescens* (PFL), the 3-hydroxy groups of **129** and **130** can be converted to the acetic acid ester or the chloroacetate, thus delivering the differently protected galactals **131–134**. Essentially the same transformations can be carried out on D-glucal, i.e. the 4-epimer of **129**. In particular, in this case a broad variety of ester functions was introduced into the 3-position of the unsaturated carbohydrate by means of lipase from *P. fluorescens*. Thereby, various differently protected glucal derivatives are accessible, carrying functional groups which can further be manipulated and differentiated by chemical and/or enzymatic methods.^{91,104}

5.1.1.2. Furanoses. The principles and the enzymes mentioned above which allow the regio- and chemoselective protection and deprotection of the various pyranoses to be carried out were also successfully applied for the enzymatic manipulation of acyl groups in furanoses. Of particular interest in this context is the finding that the five-membered rings can also be handled by the biocatalysts with a pronounced regioselectivity, although furanoses can adopt more flexible conformations with close energy in solution.



reaction conditions: acylating agent: TFEB

^asolvent: tetrahydrofuran/pyridine 4:1, ^bsolvent: tetrahydrofuran

PFL = lipase from *Pseudomonas fluorescens*



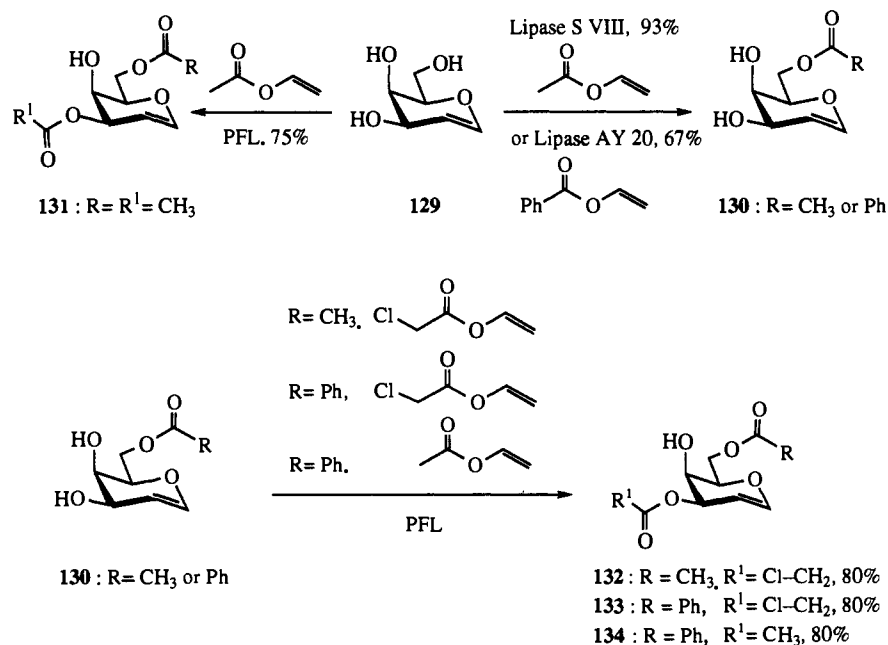
Figure 27.

The cleavage of the primary acetyl groups from the α -D-ribo-, the β -D-ribo-, the α -D-arabino-, and the β -D-2-deoxyribofuranosides 135–138 could be carried out in high yields with lipase from *C. cylindracea*⁸⁸ (Figure 29). For the 2-deoxy- α -D-ribofuranoside and the α - and the β -xylo compounds 139 and 140, respectively, which were investigated as a mixture, the hydrolysis was less selective. In the latter case the α -isomer was attacked

exclusively at the 5-position, whereas from the β -anomer only the 3-acetyl group was split off. From the peracetylated furanoses 141 and 142 the anomeric acyl group was removed with complete selectivity by means of lipase from *A. niger*. In the hydrolysis of the 5,6-diacetates of several hexofuranoses, e.g. 146, with pig liver esterase the 6-acetate was the major product.¹¹⁸ However, this unexpected regioselectivity was achieved by a preferred hydrolysis of the primary ester and subsequent acetyl migration from O-5 to O-6.

In addition to these findings, lipases are also capable of carrying out regiodiscriminating hydrolyses on furanoses which carry different acyl functions. For instance, lipase from *C. cylindracea* (CCL) also split off the *n*-butanoates from the diisopropylidene-glucopyranose 143 and the xylofuranoses 144 and 145. The enzyme from porcine pancreas could be used for this purpose as well⁹⁰ and from the acetate 143 (R = CH₃) the ester was also removed by means of acetyl esterase from oranges.^{24,43,103} In neither case were the acid labile acetals affected. It is not unexpected that in 144 the enzymes differentiate between the secondary acetate and the primary butyrate. However, if the ester of the primary alcohol is derived from pivalic acid, e.g. 145, the biocatalysts exclusively attack the 3-position, indicating that pivaloyl groups can not be hydrolyzed by the two lipases investigated (for the enzymatic hydrolysis of pivalic acid esters vide supra, Figure 22). Finally, also the ability of penicillin G acylase to hydrolyze phenylacetates was exploited to selectively deprotect furanoses. Thus, the glucose ester 143 (R = CH₂Ph) and a similarly substituted sorbose ester were cleaved enzymatically in moderate yield leaving the acetals intact.^{22–24}

The selectivity of enzyme-mediated acylations of furanoid carbohydrates parallels the findings recorded for the pyranoses. Thus, the primary hydroxyl functions of the D-ribo-, the D-xylo-, and the D-arabino



PFL = lipase from *Pseudomonas fluorescens*

Figure 28.

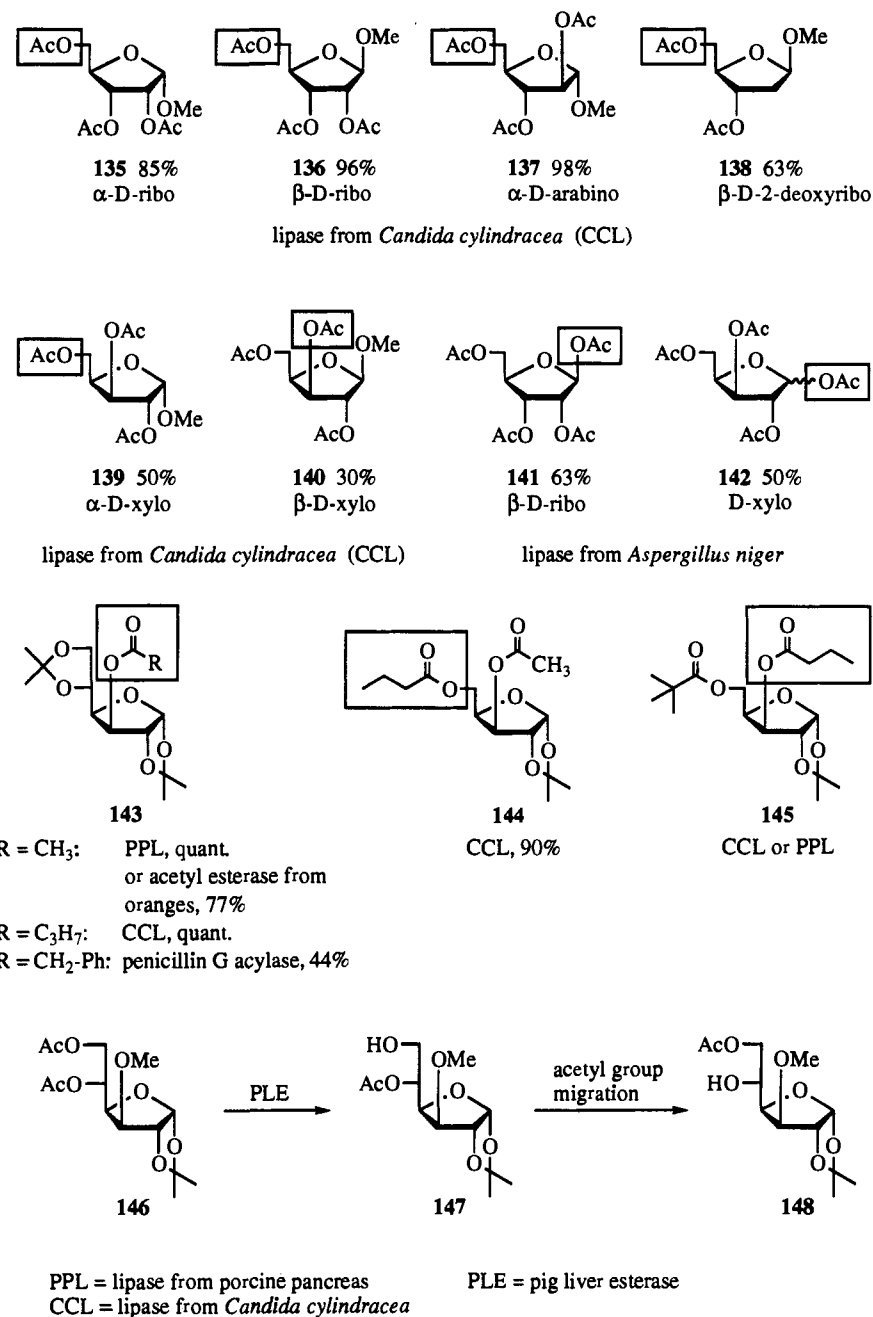


Figure 29.

glycosides 149, 150, and 151 were acetylated by tri-fluoroethyl acetate in tetrahydrofuran in the presence of lipase from porcine pancreas⁸⁶ (Figure 30). Similarly, the 1,2-isopropylideneoxyfuranose 153 could be converted to various 5-esters 154 in 60–70% yield with lipase from *C. cylindracea* (CCL).¹¹⁹ Porcine pancreatic lipase converted methyl 2-deoxyribose 152, however, to a mixture of regioisomers.⁸⁸ This drawback could be overcome by employing isopropenyl acetate as acyl donor and protease N (subtilisin) or highly stable subtilisin mutants.¹²⁰ In dimethylformamide as solvent these enzymes produced exclusively the 5-acetate in 70% yield. If the primary 5-OH group of the furanoses is already substituted, regiodiscriminating acylations of the secondary hydroxyl functions become feasible, e.g. the arabinitol ether 155 is converted predominantly to the 2- or the 3-butyrate, respectively, using lipases from different sources¹²¹ (Figure 30).

Finally, it should be mentioned, that some attempts were made to differentiate between the hydroxyl groups of fructose by enzymatic methods, but, with lipases as well as with subtilisin, only mixtures of 1- and 6-isomers were obtained.^{108,112,122} Regioselectively monosubstituted fructoses can, however, be obtained by an enzymatic approach from sucrose (vide infra, Figure 31).

5.1.2. Di- and Oligosaccharides

Only a few studies dealing with enzymatic protecting group manipulations on di- and oligosaccharides have appeared in the literature. They revealed that, in particular, the application of subtilisin together with dimethylformamide as solvent is advantageous for this purpose. As was already pointed out, the use of DMF is critical, since its dissolving ability is high enough to solubilize even highly polar polyhydroxy compounds

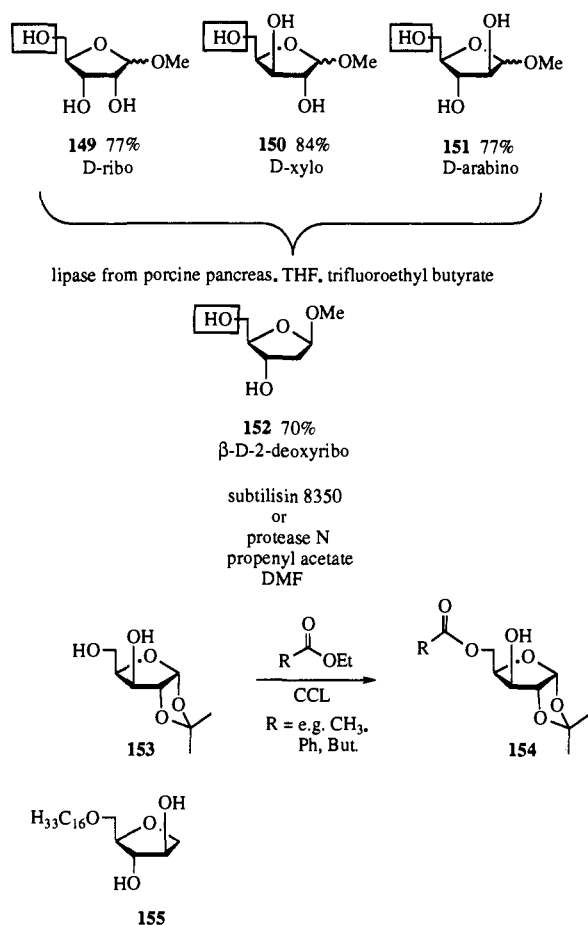


Figure 30.

(comparable experiments with pyridine as solvent failed¹⁰⁸). Subtilisin accepts several disaccharides as substrates and transfers butyric acid from trichloroethyl butyrate to the primary 6'-hydroxyl functions of the nonreducing monosaccharide of cellobiose 156 and maltobiose 157¹¹² (Figure 31).

For lactose the regioselectivity was less pronounced; however, methyl and benzyl β -D-lactosides 158 were converted to the 6'-butyrates in 71–73% yield.¹²³ Rutinose 159 in which the primary hydroxy group of the glucose moiety is blocked, is selectively acylated in the 3-position.¹²⁴ In addition, higher maltooligomers could be acylated in the 6-position of the terminal nonreducing carbohydrate, too. For instance, 6''-O-butyrylmaltotriose was isolated in 29% yield, but the corresponding tetra-, penta-, hexa- and heptamers were also substrates for the biocatalyst.¹¹² These enzymatic esterifications open a route to discriminate between the primary hydroxyl groups in di- and oligosaccharides in a convenient and straightforward way. Classical chemical one-step methods of comparable selectivity are not available for this purpose,^{84,85} and usually multistep sequences have to be carried out if the selective protection of a specific primary hydroxyl group in a di- or oligosaccharide is desired.

Due to its great commercial importance as a renewable resource, sucrose 160 has been subjected to several enzymatic hydroxyl group manipulations. This nonreducing disaccharide turned out to be a substrate for subtilisin, too.¹¹² In contrast to chemical acylations in which the most reactive OH groups are found in the 6- and the 6'-position, the enzyme selectively transfers

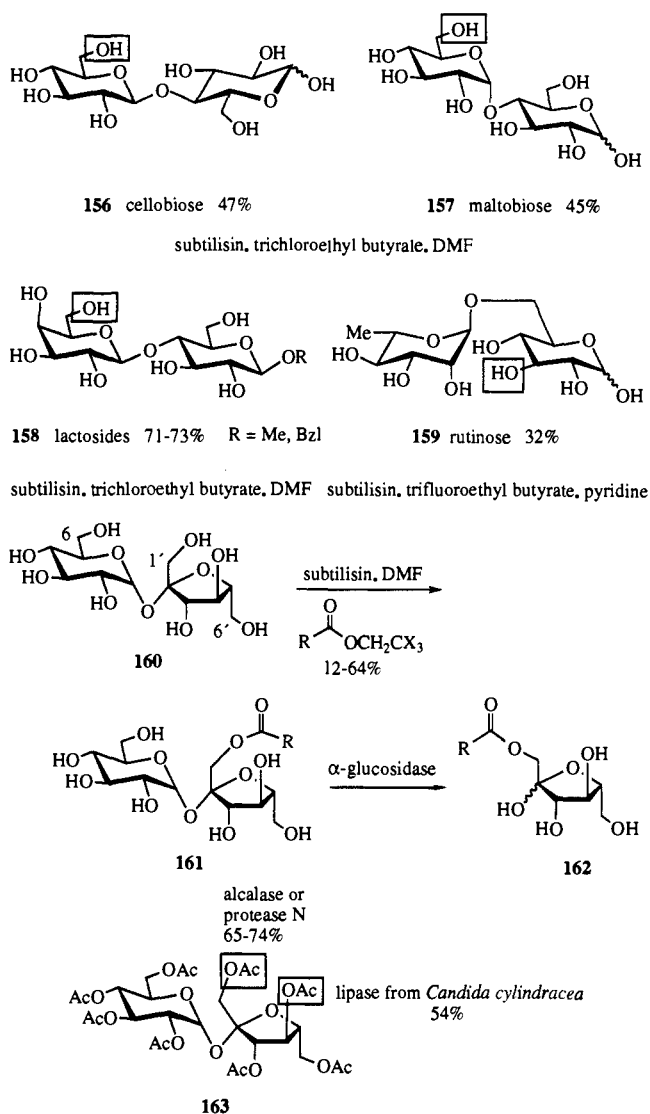


Figure 31.

various acyl functions to the 1'-alcohol^{112,122} (Figure 31). The monoacylated disaccharides 161 thereby obtained could then be further transformed enzymatically. On the one hand, with the lipase from *C. viscosum* (CVL) the free primary 6-OH group was acylated in 31% yield. On the other hand, the 1'-esters 161 are substrates for yeast α -glucosidase which hydrolyzes the glycosidic bond and thus makes the 1-O-acylfructoses 162, potentially useful as chiral synthons, available. In the direction of hydrolysis several enzymes were investigated.¹²⁵⁻¹²⁸ Depending on the biocatalyst used, acetyl groups from different positions of octaacetyl sucrose 163 could be removed selectively in useful yields. For instance, alcalase and protease N preferably attack the acetate on O-1',¹²⁸ and the lipase from *C. cylindracea* preferably liberates the OH group on C-4' of the furanoid ring^{125,126} (Figure 31).

5.1.3. Nucleosides

The directed protection of nucleoside functional groups is a fundamental problem in nucleoside and nucleotide chemistry. Although several chemical methods are available for the regioselective acylation of the nucleoside carbohydrates, enzymatic methods offer significant advantages with respect to yield, regio-

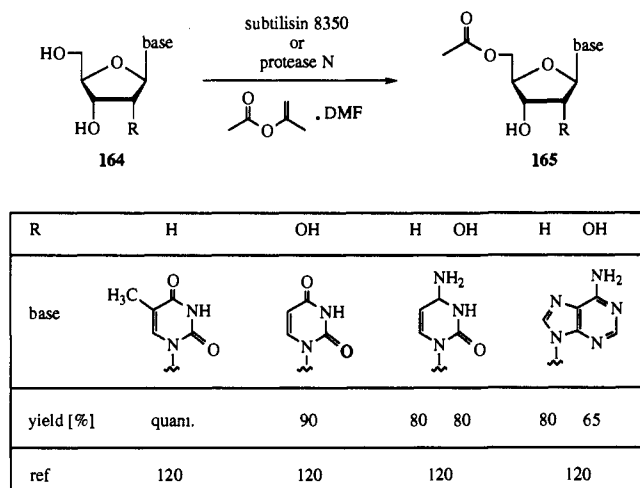


Figure 32.

lectivity, and the number of synthetic steps which have to be carried out. Earlier studies focused on the use of the dihydrocinnamoyl group as enzyme-labile nucleoside protecting function which can be removed through the agency of α -chymotrypsin.^{129,130} Although the enzyme shows an interesting tendency to attack preferably the 5'-position, this technique was not further exploited. Highly regiodiscriminating biocatalyzed acyl transfer reactions to the carbohydrate parts of various nucleosides could be carried out employing again the protease subtilisin together with dimethylformamide as solvent. In particular, a mutant of this enzyme, obtained via site-specific mutations appears to display advantageous properties. It transfers the acetyl group from isopropenyl acetate to the primary hydroxyl functions of various purine and pyrimidine nucleosides and 2'-deoxynucleosides 164 in high yields¹²⁰ (Figure 32).

Commercially available subtilisin (protease N from Amano) provided the same compounds with identical yields and selectivities; however, five times more enzyme is required for this purpose. In addition, in the transfer of butyric acid from trichloroethyl butyrate to adenosine and uridine, carried out earlier,¹¹² this biocatalyst showed inferior properties with respect to regioselectivity and yields. The selective introduction of protecting groups into the hydroxyl functions of different nucleosides can be achieved by means of lipases, too. Thus, unprotected pyrimidine and purine 2'-deoxynucleosides 164 (X = H) are converted to the 3'-O-acylated derivatives 166 and 168 in 64-82% and 54-83% yield, respectively, making use of lipase from *Pseudomonas cepacia* as the enzyme of choice and employing oxime carbonates 165 and oxime esters 167 as acyl donors^{131,132} (Figure 33).

If lipase from *Candida antarctica* is used, the carbonates are generated at the primary 5'-OH group, however.¹³³ Acylations of nucleosides with acid anhydrides in the presence of lipase from *P. fluorescens* (PFL) in DMF or DMSO as solvent proceeded with unsatisfying regioselectivity.¹³⁴ Better results were achieved with lipase from *Pseudomonas* sp.¹³⁵ However, lipase from *P. fluorescens* together with subtilisin can be applied to effect highly specific deacylations of various pyrimidine nucleosides 170¹³⁶ (Figure 34). Thus, lipase from *P. fluorescens* (PFL) preferably attacks the hexanoyl group on the secondary hydroxyl function of

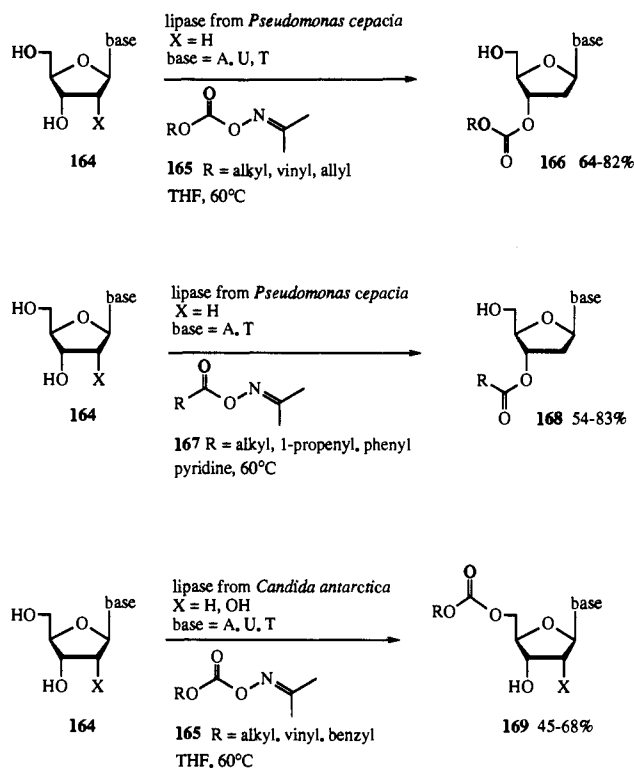


Figure 33.

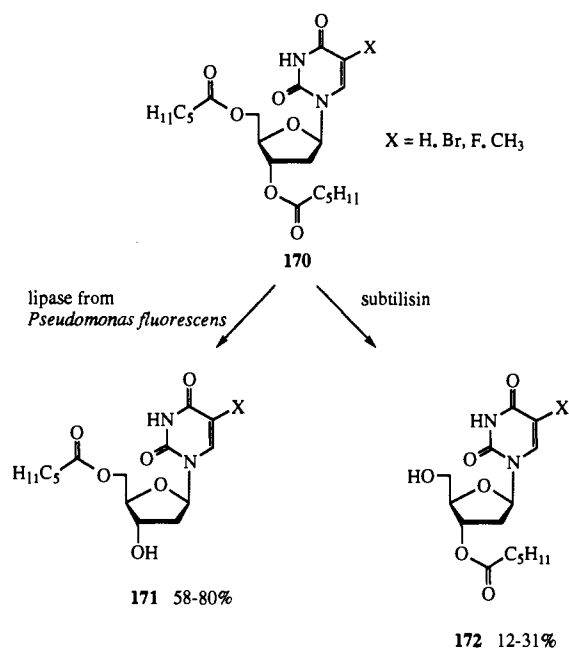


Figure 34.

the *N*-glycosides 170, giving rise to the 5-esters 171 in good yields. On the other hand, subtilisin makes the 3-esters 172 available in moderate yields. It should be noted, however, that in both cases considerable to large amounts (6-71%) of the completely deprotected nucleosides were formed.

5.1.4. Further Aglycon Glycosides

In addition to nucleosides, several other naturally occurring carbohydrate derivatives can be selectively protected/deprotected by means of enzymatic techniques. For instance, salicin 173, a wood component that contains a primary hydroxyl group located in a

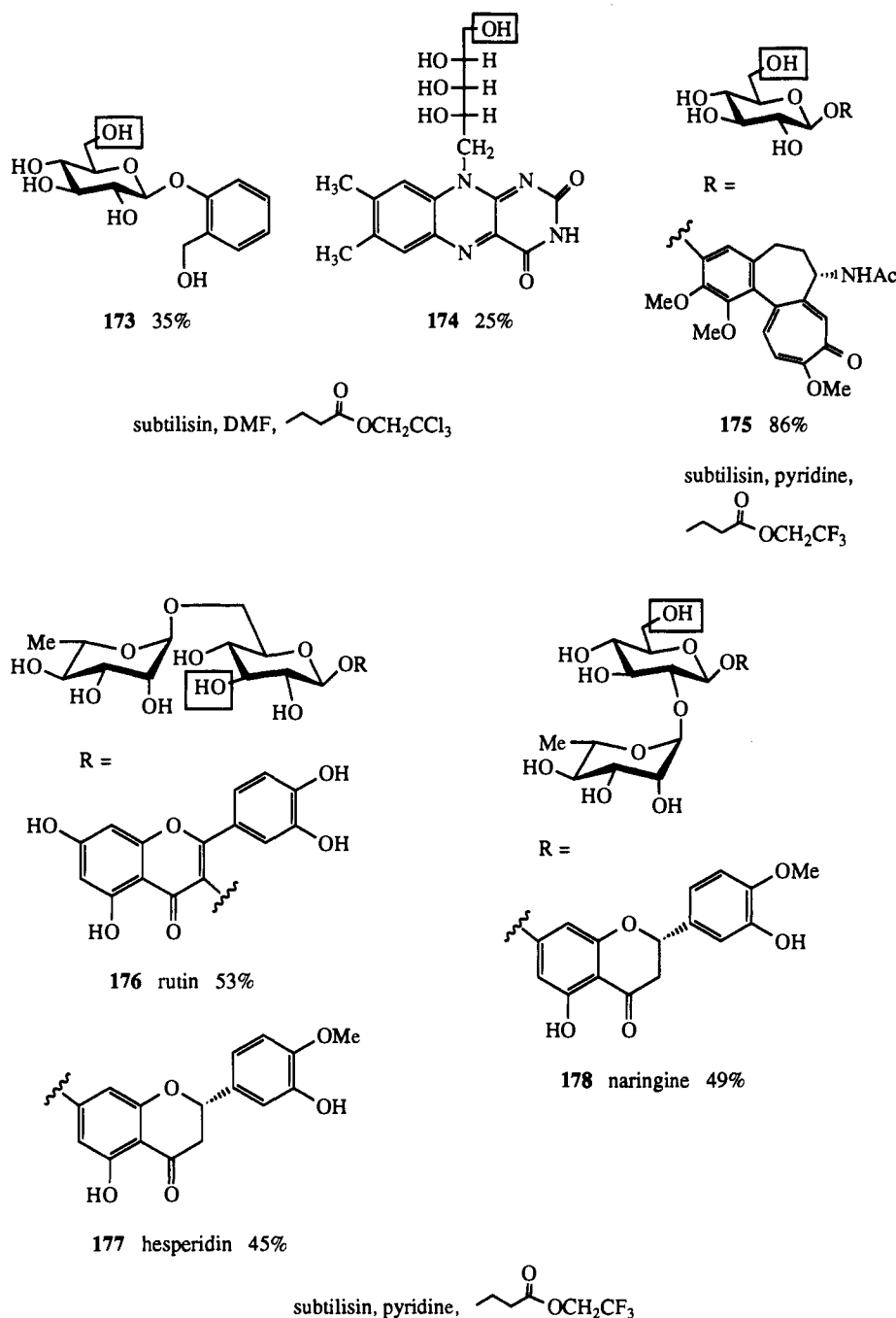


Figure 35.

glucose moiety and a second one in a benzylic position, was butyrylated exclusively at the 6-OH of the monosaccharide in 34% yield by applying subtilisin and trichloroethyl butyrate in DMF¹¹² (Figure 35). Under the same conditions, in riboflavin (vitamin B₂) 174 only the primary alcohol was esterified in 25% yield,¹¹² and colchicoside (175) as well as a thio analog was converted to the 6'-butyrates by treatment with trichloroethyl butyrate in pyridine in the presence of subtilisin.¹³⁷ Similarly, the carbohydrate parts of flavonoid disaccharides were regioselectively functionalized. Thus, for instance in the disaccharides rutin (176) and hesperidin (177) only the 3-OH group of the glucose moiety was esterified upon treatment with trifluoroethyl butyrate and subtilisin in pyridine¹²⁴ (Figure 35). Naringine (178) was converted into the 6-glucosyl ester under these conditions. In all cases the rhamnose and the phenolic

hydroxyls remained unattacked (for the protection of phenolic hydroxyl groups in flavonoids see section 5.4). For related flavonoid monosaccharides like isoquercitrin the regioselectivity is less pronounced.¹³⁸

Two impressive examples for selective enzymatic deacylations of complex substrates consist in the removal of all acetates from the peracetylated β -D-glucopyranosyl ester 179 of abscisic acid¹³⁹ and of the gibberellinic acid derivative 180,¹⁴⁰ containing one glucose tetraacetate glycosidically bound and a second one attached as an ester (Figure 36). In both cases the removal of the acetyl groups by chemical methods in particular was complicated by an undesired cleavage of the ester linkages to the glucoses. However, the four acetyl groups present in 179 could be hydrolyzed chemoselectively by means of helicase, an enzyme isolated from the seeds of *Helianthus annuus*, whereby

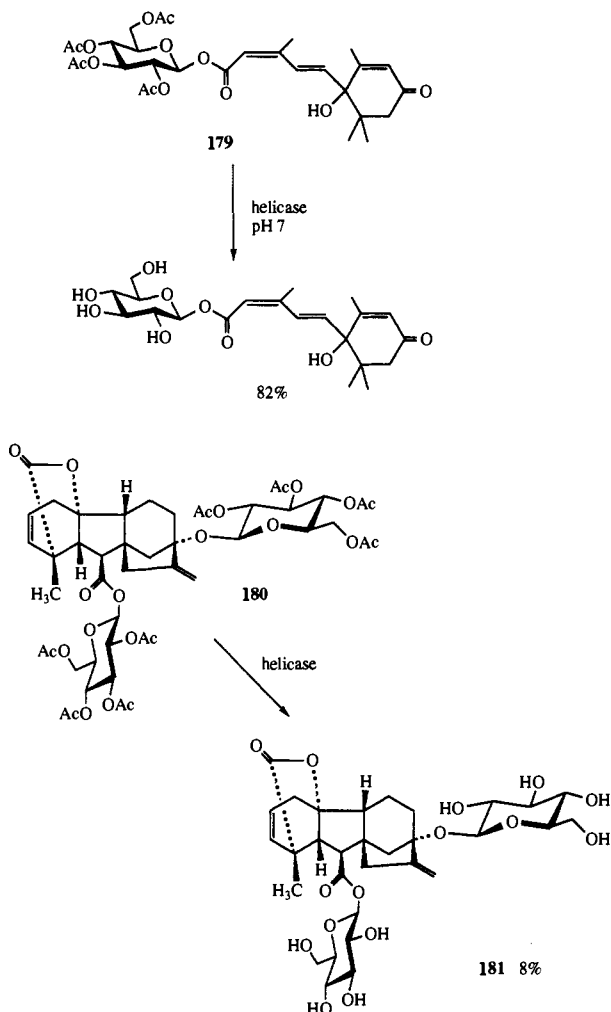


Figure 36.

the unprotected glucose ester was formed in 82% yield without destroying the ester bond between abscisic acid and glucose. Similarly, the enzyme removed all acetates from 180. In this case the yield reached only 8%; it should, however, be kept in mind that eight acetic acid esters had to be cleaved in the enzymatic process and that the aglycon is rather complex.

In conclusion, the various enzyme-mediated protecting group manipulations carried out on numerous saccharide derivatives indicate that biocatalysts can advantageously be used in the protecting group chemistry of carbohydrates. In particular, subtilisin and several lipases from different sources (from porcine pancreas, from *C. cylindracea*, from *A. niger*, from *C. viscosum*, from *M. javanicus*, from *P. fluorescens*, and from wheat germ) allow the chemo- and regioselective acylation and deprotection of various saccharides to be carried out, whose structures differ widely. A general principle that emerges from these studies is that the enzymes exhibit a predominant preference toward primary hydroxyl groups. If these functional groups are not present or protected, the biocatalysts are capable of selectively manipulating secondary hydroxyl groups or esters thereof. In the introduction and removal of acyl groups, the regioselectivity displayed by the enzymes often parallels the findings recorded for classical chemical transformations, although it is often significantly higher. Furthermore, in several cases regioselectivities were observed in the biocatalyzed

processes which cannot or only hardly be achieved by means of classical chemical methods. Finally, it should be realized that subtilisin and the lipases are capable of introducing specific acyl groups into the carbohydrates which can afterward be removed selectively by different enzymatic or nonenzymatic methods.

5.2. Protection of Polyhydroxylated Alkaloids

The plant alkaloid castanospermine (182) and the related piperidine alkaloid 1-deoxynojirimycin (187), like several other polyhydroxylated octahydroindolizidines, piperidines, and pyrrolidines, are potent glycosidase inhibitors. These nitrogen bases are of considerable interest for the study of biosynthetic processes and, in addition, castanospermine and some of its derivatives may be of clinical value as antineoplastic agents and as drugs in the treatment of AIDS. In the light of the analogy between the structures of these alkaloids and glucose, some of the above mentioned enzymatic methods for the selective functionalization of carbohydrates were applied to prepare several acyl derivatives of 182 and 187. Thus, subtilisin transfers the acyl moieties from several activated esters to the 1-OH group of the bicyclic base in moderate to high yields^{141,142} (Figure 37). Again, pyridine had to be used as solvent for the polyhydroxy compound. The monoesters 183 obtained by this technique, like the monoesters of hexoses, could subsequently be dissolved in THF and were further acylated by means of different enzymes. The 1-phenylacetate, for instance, was converted regioselectively to the 6-butyrate 184 by subtilisin in low yield. On the other hand, the 1,7-dibutyrate 185 was obtained from 183 in a process catalyzed by lipase from *C. viscosum* (CVL). Finally, the 1-ester was removed from 185 by subtilisin in aqueous solution to deliver the 7-butanate 186 in 64% yield.

In contrast to castanospermine, 1-deoxynojirimycin 187 contains a primary hydroxyl group as well as a much more nucleophilic secondary amino function. If a small excess of trifluoroethyl butyrate is employed, subtilisin converts this alkaloid preferably into the 6-monoester 188¹⁴¹ (Figure 37). However, with 6 equiv of the acylating agent, the 2,6-diester 189 is formed in 77% yield. 189 may be subsequently deacylated regioselectively at the 6-position by means of several different enzymes.

It should be noted that under the conditions of the enzymatic acylation the amino group is not derivatized, an observation which has also been made in related cases,^{142,143} e.g. N-terminally deprotected serine peptides.

5.3. Protection of Steroids

Enzymatic acyl transfer reactions also are practical processes for the acylation of hydroxyl groups in steroids. The lipase from *C. viscosum* (CVL) for instance selectively transfers butyric acid from trifluoroethyl butyrate to equatorial (β) C-3-alcoholic functions being present in a variety of sterols, e.g. 190 and the respective 5,6-didehydro compound 191¹⁴⁴ (Figure 38). Axially oriented alcohols at C-3 and secondary alcohols at C-17 or in the sterol side chains are not derivatized. In addition to the equatorial

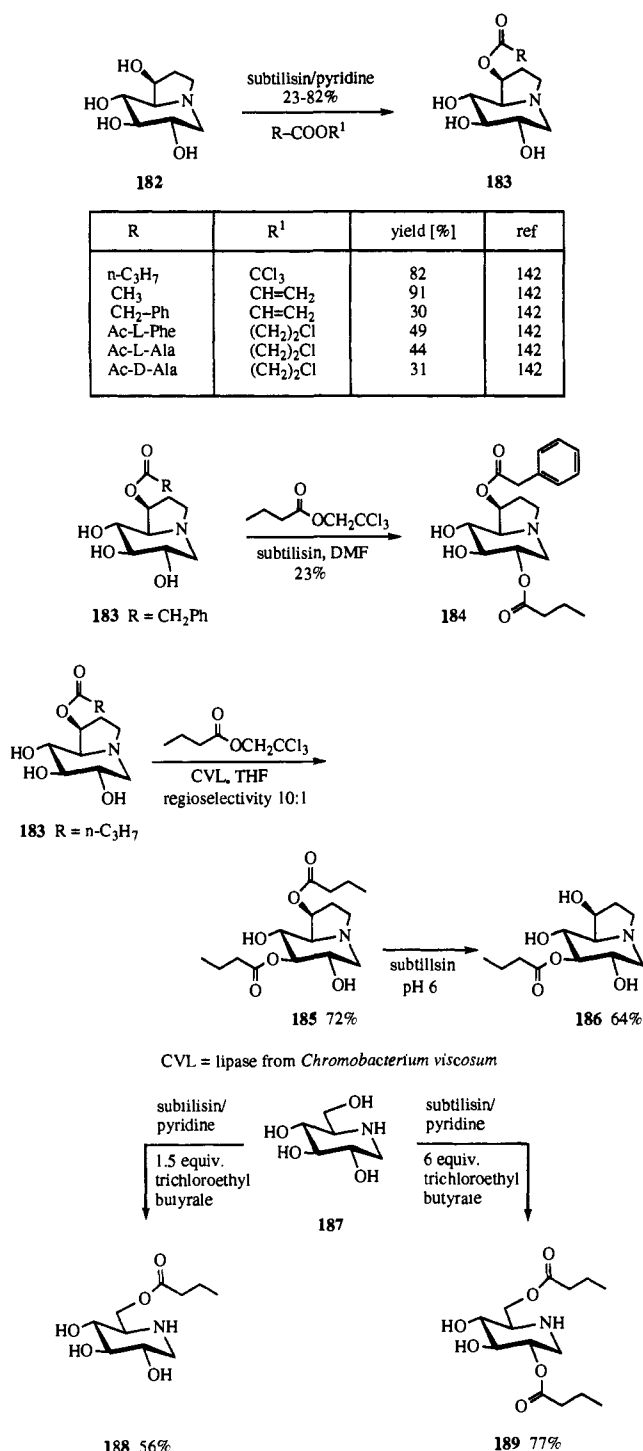


Figure 37.

alcohols, the compounds being accepted as substrates by the lipase must have the A/B-ring fusion in the trans configuration. In the B ring a double bond is tolerated, but not in the A ring. In contrast, subtilisin does not recognize the hydroxyl group at C-3 of the steroid nucleus, but rather transfers the acyl moiety to alcoholic groups in the 17-position or in the side chains (Figure 38). Changes in the A or in the B ring do not dramatically influence the selective mode of action of this biocatalyst. Thus, using these two enzymes, the completely regioselective protection of either alcoholic group in several steroid diols is possible. This feature opened a route to a new chemoenzymatical process for the oxidation of selected positions of the steroid

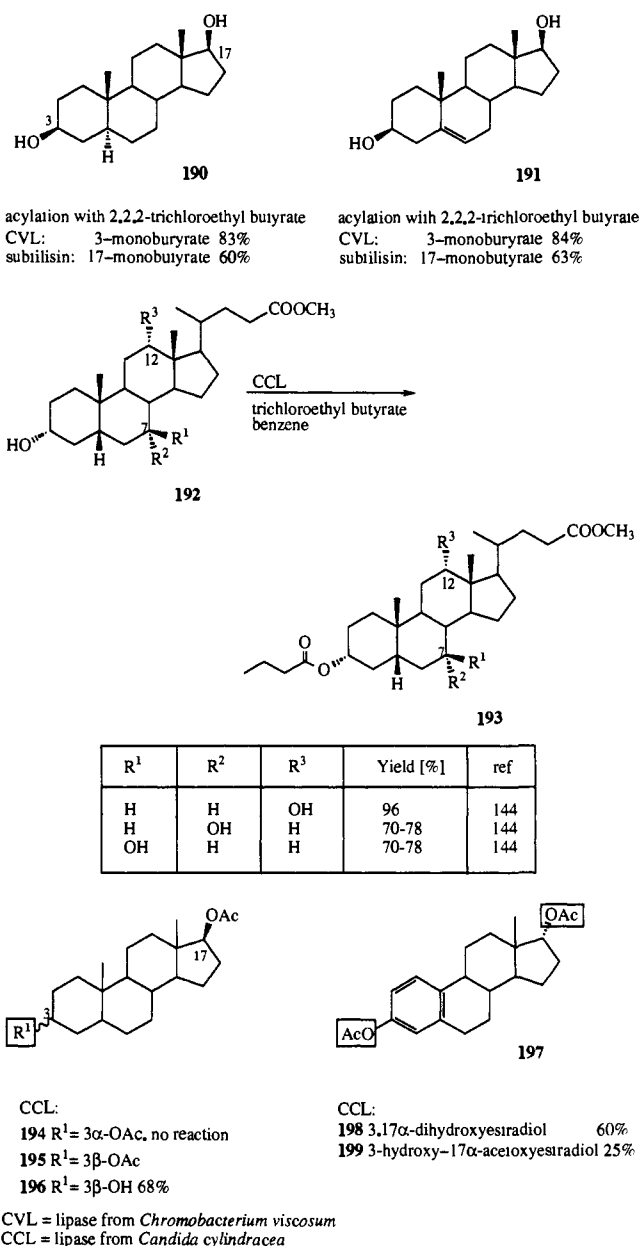


Figure 38.

framework. Chemoenzymatic approaches of this type are expected to provide attractive alternatives to the currently utilized enzymatic oxidation of steroids by hydroxysteroid dehydrogenases.

A further biocatalyst comes into play, when bile acids serve as starting materials, e.g. deoxycholic acid methyl ester 192 (R¹ = R² = H, R³ = OH).¹⁴⁵ The cis configuration of the A/B-ring fusion prevents the application of lipase from *C. viscosum* (CVL), and the aliphatic chain hinders the esterification of the 12 α -hydroxyl group by subtilisin. The lipase from *C. cylindracea* (CCL) has proven to be the most suitable biocatalyst for the enzymatic acylation of bile acids. In hydrophobic solvents, i.e. hexane, toluene, butyl ether, benzene, etc. (except acetone) and by employing trichloroethyl butanoate as acyl donor, the 3 α -O-butanoyldeoxycholic acid methyl ester 193 (R¹ = R² = H, R³ = OH) is formed in 96% yield without any byproducts, suggesting that the enzyme is ineffective toward 12 α -OH. In addition, the 7 α -OH and the 7 β -OH, present in 192 (R¹ = R³ = H, R² = OH) and 192

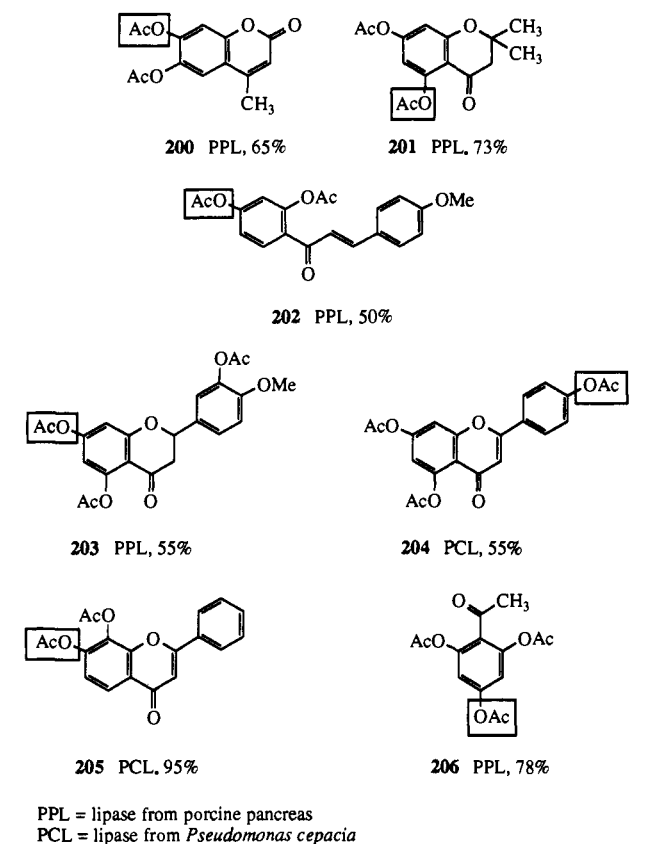


Figure 39.

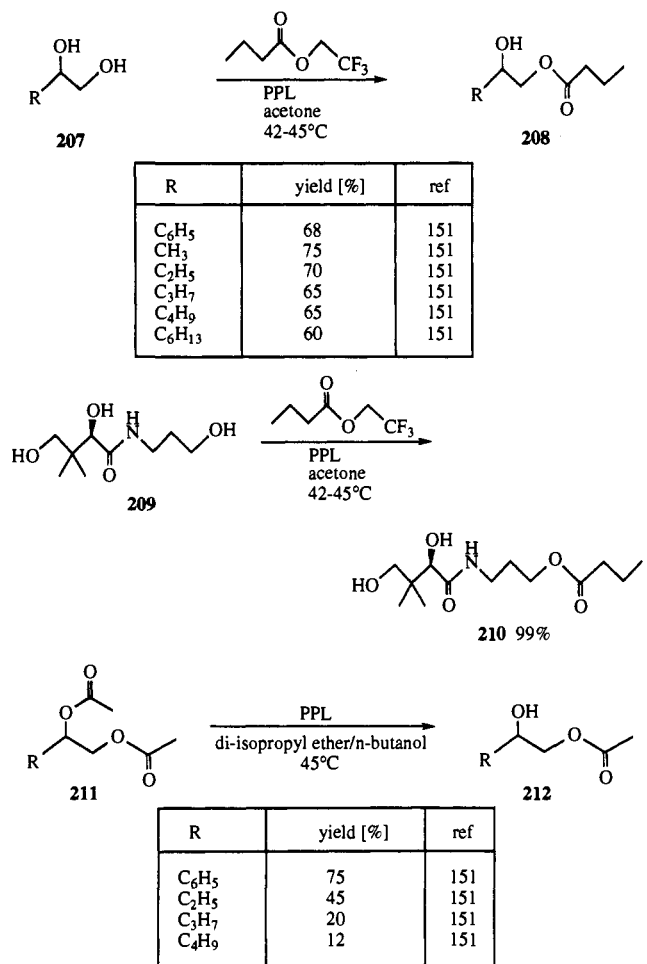
(R¹ = OH, R² = R³ = H) are not esterified by the enzyme, in both cases, also the 3-butanoate is formed (Figure 38).

The saponification of steroid esters can be steered with *C. cylindracea* lipase.¹⁴⁶ This process occurs in the presence of octanol in organic solvents and is characterized by a pronounced stereospecificity and regioselectivity. Thus, the 3 α -esters of 3 α ,17 β -diacetoxy steroid 194 resisted liberation, whereas the 3 β -isomer 195 is transformed to the corresponding alcohol 196 in 68% yield. The 17 α -acetate of 3,17 α -diacetoxy estradiol 197 is also saponified, but at a slower rate than the C-3 acetate (Figure 38).

5.4. Protection of Phenolic Hydroxy Groups

Polyphenolic compounds occur widely distributed in nature and may possess a variety of interesting biological properties, e.g. antibiotic, antiviral, and antitumor activity. The synthesis and further elaboration of these compounds often requires the selective protection or deprotection of specific phenolic hydroxy groups. To achieve this goal, the methods highlighted above for the various aliphatic polyols can be applied successfully.

Thus, the coumarin (200), the chromanone (201), the chalcone (202), the flavanone (203), as well as several flavones, e.g. 204 and 205, were regioselectively deacylated by employing different lipases in organic solvents (Figure 39). Porcine pancreatic lipase (PPL) predominantly attacks one of the phenolic acetates present in 200–203 with good to high regioselectivity and makes the respective selectively protected compounds available in good yields.¹⁴⁷ The flavone acetates 204 and



PPL = lipase from porcine pancreas

Figure 40.

205 can be partially deacylated with high regioselectivity by transesterification using lipase from *P. cepacia* (PCL) and *n*-butyl alcohol in THF.^{148,149} However, in other cases the positional specificity displayed by the enzyme was less pronounced. This technique has allowed for an efficient construction of a selectively *O*-methylated flavonoid.¹⁴⁹

In addition, aryl alkyl ketones which are important starting materials for the synthesis of polyphenolic natural products may be manipulated selectively by making use of an enzymatic saponification.¹⁴⁷ In general, the sterically better accessible ester groups are cleaved, as for instance in 206.

5.5. Protection of Glycerol Derivatives and Related Polyols

Selectively functionalized polyols, e.g. glycerol esters occur in a variety of natural products and are important starting materials for the synthesis of more complex target compounds. Therefore, the selective protection and deprotection of their hydroxyl groups is of considerable interest.

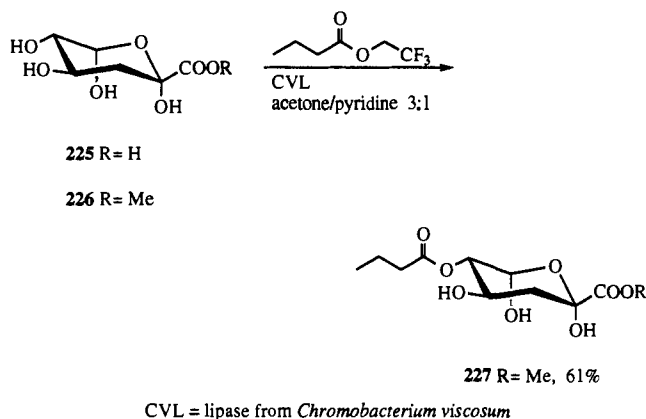


Figure 42.

6. Outlook

During the last decade substantial progress was achieved in the development of enzymatic protecting group techniques. In particular, it was demonstrated that these methods offer viable alternatives to classical chemical approaches. Not only do the biocatalyzed transformations complement the arsenal of nonenzymatically removable protecting groups, but in many cases they additionally offer the opportunity to carry out useful functional group interconversions with selectivities which cannot or only hardly be matched by classical chemical techniques. However, the overwhelming majority of the investigations carried out in this area has restricted itself to the study of the protection and the deprotection of model compounds. Complex synthetic schemes were nearly generally avoided. Whereas this appears to be particularly true for the carbohydrate field (to date no di- or oligosaccharide has been built up employing enzymatic protecting group techniques), noticeable examples which demonstrate the capacity of these biocatalyzed processes were recorded in peptide chemistry. The data and observations highlighted above, however, provide a solid basis for the application of biocatalysts in the handling of protecting group problems in complex multistep syntheses. Therefore, in the near future, the application of enzymes for this purpose can be expected.

On the other hand, the use of biocatalysts in protecting group chemistry in the sense of a general method deserves and is certainly awaiting a further intensive development. Numerous applications of the known enzymes appear to be possible in all areas of preparative chemistry. In addition, the use of catalytic proteins which have not yet been applied to carry out protecting group manipulations and of biocatalysts unknown today will create new opportunities for improved organic syntheses.

7. References

- (1) Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley and Sons, New York, 1991.
- (2) Kunz, H.; Waldmann, H. *Comprehensive Organic Synthesis*; Trost, B. M., Fleming, I., Winterfeldt, E., Eds.; Pergamon Press, Oxford, 1991; Vol. 6, pp 631-701.
- (3) Waldmann, H. *Kontakte (Darmstadt)* 1991, 2, 33-54.
- (4) Reidel, A.; Waldmann, H. *J. Prakt. Chem.* 1993, 335, 109-127.
- (5) Glass, J. D. In *The Peptides*; Udenfield, S., Meienhofer, J., Eds.; Academic Press: San Diego, 1987; pp 167-184.

- (6) Wunsch, E.; Deffner, M.; Deimer, K.-H.; Jaeger, E.; Stelzel, P.; Thamm, P.; Wendberger, G. *Methoden Org. Chem. (Houben-Weyl)*; Thieme: Stuttgart, 1974; Vols. XV/I and II.
- (7) Holley, R. W. *J. Am. Chem. Soc.* 1955, 77, 2552-2553.
- (8) Widmer, F.; Bayne, S.; Houen, G.; Moss, B. A.; Rigby, R. D.; Whittaker, R. G.; Johansen, J. T. In *Peptides 1984*, Ragnarsson, U., Ed.; Almqvist & Wiksell: Stockholm 1985; pp 193-200.
- (9) Glass, J. D.; Meyers, C.; Schwartz, I. L.; Walter, R. In *Proc. 18th Europ. Pept. Symp.*, Wolman, V., Ed.; Wiley and Sons: New York, 1974; pp 141-151.
- (10) Meyers, C.; Glass, J. D. *Proc. Natl. Acad. Sci. U.S.A.* 1975, 72, 2193-2196.
- (11) Meyers, C.; Glass, J. D. In *Peptides: Chemistry, Structure and Biology*; Walter, R., Meienhofer, J., Eds.; Ann Arbor Science Publishers: Ann Arbor, 1975; pp 325-331.
- (12) Patthy, L.; Smith, E. L. *J. Biol. Chem.* 1975, 250, 557-564.
- (13) Glass, J. D.; Pelzig, M.; Pande, C. S. *Int. J. Pept. Prot. Res.* 1979, 13, 28-34.
- (14) Pande, C. S.; Pelzig, M.; Glass, J. D. *Proc. Natl. Acad. Sci. U.S.A.* 1980, 77, 895-899.
- (15) Glass, J. D.; Pande, C. S. In *Peptides: Structure and Function*; Hruby, V., Rich, D. H., Eds.; Pierce Chem. Co.: Rockford, Ill, 1983; pp 203-206.
- (16) Blake, J.; Li, C. H. *Proc. Acad. Sci. U.S.A.* 1983, 80, 1556-1559.
- (17) Widmer, F.; Breddam, K.; Johansen, J. T. In *Peptides 1980*; Brunfeldt, K., Ed.; Scriptor: Copenhagen, 1981; pp 46-55.
- (18) Wunsch, E.; Moroder, L.; Göhring, W.; Thamm, P.; Scharf, R. *Hoppe Seyler's Z. Physiol. Chem.* 1981, 362, 1285-1287.
- (19) Wang, Q.-C.; Fei, J.; Cui, D.-F.; Zhu, S.-G.; Xu, L.-G. *Biopolymers* 1986, 25, 109-114.
- (20) Fuganti, C.; Grasselli, P.; Casati, P. *Tetrahedron Lett.* 1986, 27, 3191-3194.
- (21) Waldmann, H. *Tetrahedron Lett.* 1988, 29, 1131-1134.
- (22) Waldmann, H. *Liebigs Ann. Chem.* 1988, 1175-1180.
- (23) Waldmann, H.; Braun, P.; Kunz, H. *Biomed. Biochim. Acta* 1991, 50, 243-248.
- (24) Waldmann, H.; Heuser, A.; Braun, P.; Schulz, M.; Kunz, H. In *Microbial Reagents in Organic Synthesis*; Servi, S., Ed.; Kluwer: Dordrecht, 1992; pp 113-122.
- (25) Hermann, P. *Wis. Z. Univ. Halle* 1987, 36, 17-29.
- (26) Hermann, P. *Biomed. Biochim. Acta* 1991, 50, 19-31.
- (27) Greiner, G.; Hermann, P. In *Peptides 1990*; Giralt, E.; Andreu, D., Eds.; Escam: Amsterdam, 1991; pp 227-278.
- (28) Margolin, A. L.; Svedas, V. K.; Berezin, I. V. *Biochim. Biophys. Acta* 1980, 616, 283-289.
- (29) Didziapetris, R. J.; Svedas, V. K. *Biomed. Biochim. Acta* 1991, 50, 237-242.
- (30) Didziapetris, R.; Drabnig, B.; Schellenberger, V.; Jakubke, H.-D.; Svedas, V. *FEBS Lett.* 1991, 287, 31-33.
- (31) Schütz, H. J.; Wandrey, C.; Leuchtenberger, W. Abstracts of the Ninth Engineering Foundation Conference on Enzyme Engineering, New York, 1987; Chapter I, p 11.
- (32) Stoineva, I.; Galunsky, B.; Lozanov, V.; Ivanov, I.; Petkov, D. *Tetrahedron* 1992, 48, 1115-1122.
- (33) Balduino, E.; Fuganti, C.; Servi, S.; Tagliani, A.; Terreni, M. In *Microbial Reagents in Organic Synthesis*; Servi, S., Ed.; Kluwer: Dordrecht, 1992; pp 175-188.
- (34) Suyama, T.; Tagoda, T.; Kanao, S. *Yakugaku Zasshi*, 1965, 85, 279.
- (35) Pessina, A.; Lüthi, P.; Luisi, P. L.; Prenosil, J.; Zhang, Y. *Helv. Chim. Acta* 1988, 71, 631-641.
- (36) Widmer, F.; Ohno, M.; Smith, M.; Nelson, N.; Anfinson, C. B. In *Peptides 1982*; Blaha, P., Malon, P., Eds.; de Gruyter: Berlin, 1983; pp 375-379.
- (37) Matsumura, E.; Shin, T.; Murao, S.; Sakaguchi, M.; Kawano, T. *Angew. Biol. Chem.* 1985, 49, 3643-3645.
- (38) Brtnik, F.; Barth, T.; Jost, K. *Collect. Czech. Chem. Commun.* 1981, 46, 1983-1989.
- (39) See for instance: Savidge, T. A. In *Biotechnology of Industrial Antibiotics*; Vandamme, E. J., Ed.; Marcel Dekker: New York, 1984.
- (40) Shjewale, J. G.; Deshpande, B. S.; Sudhakaran, V. K.; Ambedkar, S. S. *Process Biochem. Int.* 1990, 97-103.
- (41) Balduino, E.; Faiardi, D.; Fuganti, C.; Grasselli, P.; Lazzarini, A. *Tetrahedron Lett.* 1988, 29, 4623-4624.
- (42) Ernest, I.; Kaldova, J.; Fröstl, W. Eur. Pat. Appl. 126709, November 1984. See also: Hungerbühler, E.; Biollaz, M.; Ernest, I.; Kaldova, J.; Lang, M.; Schneider, P.; Sedelmeier, G. In *New Aspects of Organic Chemistry I*; Yoshida, Z., Shiba, T., Oshiro, Y., Eds.; VCH: Weinheim, 1989; pp 419-451.
- (43) Waldmann, H.; Heuser, A.; Reidel, A. *Synlett* 1994, 65-67.
- (44) Walton, E.; Rodin, J. O.; Stammer, C. H.; Holly, F. W. *J. Org. Chem.* 1962, 27, 2255-2257.
- (45) Kloss, G.; Schröder, E. *Hoppe-Seyler's Z. Physiol. Chem.* 1964, 336, 248-256.
- (46) Xaus, N.; Clapes, P.; Bardaji, E.; Torres, J. L.; Jorba, X.; Mata, J.; Valencia, G. *Biotechnol. Lett.* 1989, 11, 393-396.
- (47) Hayward, C. F.; Offord, R. E. In *Peptides 1969*; Scoffone, E., Ed.; North-Holland Publ.: Amsterdam, 1971; pp 116-120.

- (48) Anantharamaiah, G. M.; Roeske, R. W. In *Peptides: Synthesis, Structure, Function*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: Rockford; 1982; pp 45-47.
- (49) Ohno, M.; Anfinsen, C. B. *J. Am. Chem. Soc.* 1970, 92, 4098-4102.
- (50) Royer, G.; Anantharamaiah, G. M. *J. Am. Chem. Soc.* 1979, 101, 3394-3396.
- (51) Royer, G. P.; Hsiao, H. Y.; Anantharamaiah, G. M. *Biochimie* 1980, 62, 537-541.
- (52) Steinke, D.; Kula, M.-R. *Angew. Chem.* 1990, 102, 1204-1206; *Angew. Chem., Int. Ed. Engl.* 1990, 29, 1139-1141.
- (53) Steinke, D.; Kula, M.-R. *Biomed. Biochim. Acta* 1991, 50, 143-148.
- (54) Aleksiev, B.; Schamlan, P.; Widenov, G.; Stoev, S.; Zachariev, S.; Golovinsky, E. *Hoppe-Seyler's Z. Physiol. Chem.* 1981, 362, 1323-1329.
- (55) Chen, S.-T.; Hsiao, S.-C.; Chang, C.-H.; Wang, K.-T. *Synth. Commun.* 1992, 22, 391-398.
- (56) Chen, S.-T.; Chen, S.-Y.; Hsiao, S.-C.; Wang, K.-T. *Biomed. Biochim. Acta* 1991, 50, 181-186.
- (57) Chen, S.-T.; Wu, S.-H.; Wang, K.-T. *Int. J. Pept. Protein Res.* 1991, 37, 347-350.
- (58) Hermann, P.; Salewski, L. In *Peptides 1982*; Blaha, K., Malon, P., Eds.; de Gruyter: Berlin, 1983; pp 399-402.
- (59) Hermann, P.; Baumann, H.; Herrnstadt, Ch.; Glanz, D. *Amino Acids* 1992, 3, 105-118.
- (60) Dudek, S.; Friebe, S.; Hermann, P. *J. Chromatogr.* 1990, 520, 333-338.
- (61) Braun, P.; Waldmann, H.; Vogt, W.; Kunz, H. *Synlett* 1990, 105-107.
- (62) Braun, P.; Waldmann, H.; Vogt, W.; Kunz, H. *Liebigs Ann. Chem.* 1991, 165-170.
- (63) Waldmann, H.; Kunz, H.; Braun, P. Unpublished results.
- (64) Braum, G.; Braun, P.; Kowalczyk, D.; Kunz, H. *Tetrahedron Lett.* 1993, 34, 3111-3114.
- (65) Margolin, A.; Klibanov, A. M. *J. Am. Chem. Soc.* 1987, 109, 3802-3804.
- (66) Chen, C.-S.; Sih, C. J. *Angew. Chem.* 1989, 101, 711-724; *Angew. Chem., Int. Ed. Engl.* 1989, 28, 695-708.
- (67) Braun, P.; Waldmann, H.; Kunz, H. *Synlett* 1992, 39-40; *Bioorg. Med. Chem.* 1993, 1, 197-207.
- (68) Schulz, M.; Hermann, P.; Kunz, H. *Synlett* 1992, 37-38.
- (69) Cantacuzene, D.; Attal, S.; Bay, S. *Bioorg. Med. Chem. Lett.* 1991, 197-200.
- (70) Cantacuzene, D.; Attal, S.; Bay, S. *Biomed. Biochim. Acta* 1991, 50, 231-236.
- (71) Ishii, H.; Unabashi, K.; Mimura, Y.; Inoue, Y. *Bull. Chem. Soc. Jpn.* 1990, 63, 3042-3043.
- (72) Attal, S.; Bay, S.; Cantacuzene, D. *Tetrahedron* 1992, 48, 9251-9260.
- (73) Fischer, A.; Schwarz, A.; Wandrey, C.; Bommarius, A.; Knaup, G.; Drauz, K. *Biomed. Biochim. Acta* 1991, 50, 169-174.
- (74) Glass, J.; Pelzig, M. *Proc. Natl. Acad. Sci. U.S.A.* 1977, 74, 2739-2741.
- (75) Chen, S.-T.; Wang, K.-T. *Synthesis* 1987, 581-582.
- (76) Chen, S.-T.; Wang, K.-T. *J. Chem. Res. (S)* 1987, 308-309.
- (77) Xaus, N.; Clapés, P.; Bardaji, E.; Torres, J. L.; Jorba, X.; Mata, J.; Valencia, G. *Tetrahedron* 1989, 45, 7421-7426.
- (78) Wu, S.-H.; Chu, F.-Y.; Chang, C.-H.; Wang, K.-T. *Tetrahedron Lett.* 1991, 32, 3529-3530.
- (79) Jakubke, H.-D.; Kuhl, P.; Kónnecke, A. *Angew. Chem.* 1985, 97, 79-87; *Angew. Chem., Int. Ed. Engl.* 1985, 24, 85-93.
- (80) Wieland, T. *Acta Chim. Hung. (Budapest)* 1965, 44, 5-9.
- (81) von dem Bruch, K.; Kunz, H. *Angew. Chem.* 1990, 102, 1520-1522; *Angew. Chem., Int. Ed. Engl.* 1990, 29, 1457-1460.
- (82) Drueckhammer, D. G.; Hennen, W. J.; Pederson, R. L.; Barbas, C. F., III; Gautheron, C. M.; Krach, T.; Wong, C.-H. *Synthesis* 1991, 499-525.
- (83) Faber, K.; Riva, S. *Synthesis* 1992, 895-910.
- (84) Haines, A. H. *Adv. Carbohydr. Chem. Biochem.* 1976, 33, 11-109; 1981, 39, 13-70.
- (85) Stanek, J. *Top. Curr. Chem.* 1990, 154 (Thiem, J., Ed.), 209-256.
- (86) Shaw, J.-F.; Klibanov, A. M. *Biotechnol. Bioeng.* 1987, 29, 648-651.
- (87) Fink, A. L.; Hay, G. W. *Can. J. Biochem.* 1969, 47, 353-359.
- (88) Hennen, W. J.; Sweers, H. M.; Wang, Y.-F.; Wong, C.-H. *J. Org. Chem.* 1988, 53, 4939-4945.
- (89) Sweers, H. M.; Wong, C.-H. *J. Am. Chem. Soc.* 1986, 108, 6421-6422.
- (90) Kloosterman, M.; Moosmuller, E. W.; Schoemaker, H. E.; Meijer, E. M. *Tetrahedron Lett.* 1987, 28, 2989-2992.
- (91) Holla, E. W. *Angew. Chem.* 1989, 101, 222-223; *Angew. Chem., Int. Ed. Engl.* 1989, 28, 220-221.
- (92) Matsui, T.; Kita, Y.; Matsushita, Y.; Nakayama, M. *Chem. Exp.* 1992, 7, 45-48.
- (93) Therisod, M.; Klibanov, A. M. *J. Am. Chem. Soc.* 1987, 109, 3977-3981.
- (94) Koosterman, M.; De Nijs, M. P.; Weijnen, J. G.; Schoemaker, H. E.; Meijer, E. M. *J. Carbohydr. Chem.* 1989, 8, 333-341.
- (95) Zemek, J.; Kucar, S.; Anderle, D. *Collect. Czech. Chem. Commun.* 1987, 52, 2347-2352.
- (96) Csuk, R.; Glänzer, B. *J. Z. Naturforsch.* 1988, 43b, 1355-1357.
- (97) Ballesteros, A.; Bernabe, M.; Cruzado, C.; Martin-Lomas, M.; Otero, C. *Tetrahedron* 1989, 45, 7077-7082.
- (98) Holla, E. W.; Sinnwell, V.; Klaffke, W. *Synlett* 1992, 413-414.
- (99) Tomic, S.; Tomasic, J.; Sesartic, L.; Ladesic, B. *Carbohydr. Res.* 1987, 161, 150-155.
- (100) Tomic, S.; Ljevacovic, D.; Tomasic, J. *Carbohydr. Res.* 1989, 188, 222-227.
- (101) Tomic, S.; Tresec, A.; Ljevakovic, D.; Tomasic, J. *Carbohydr. Res.* 1991, 210, 191-198.
- (102) Ljevacovic, D.; Tomic, S.; Tomasic, J. *Carbohydr. Res.* 1992, 230, 107-115.
- (103) Waldmann, H.; Heuser, A. *Bioorg. Med. Chem.*, in press.
- (104) Holla, E. W. *J. Carbohydr. Chem.* 1990, 9, 113-119.
- (105) Tamura, M.; Kinomura, K.; Tada, M.; Nakatsuka, T.; Okai, H.; Fukui, S. *Agric. Biol. Chem.* 1985, 49, 2011-2023.
- (106) Klibanov, A. M. *CHEMTECH* 1986, 354-359.
- (107) Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* 1985, 82, 3192-3196.
- (108) Therisod, M.; Klibanov, A. M. *J. Am. Chem. Soc.* 1986, 108, 5638-5640.
- (109) Forstner, M.; Freytag, K.; Paschke, E. *Carbohydr. Res.* 1989, 193, 294-295.
- (110) (a) Wang, Y.-F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* 1988, 110, 7200-7205. (b) Pulido, R.; Lopez Ortiz, F.; Gotor, V. *J. Chem. Soc., Perkin Trans. 1* 1992, 2891-2898.
- (111) Ghogare, A.; Kumar, G. Sudesh *J. Chem. Soc., Chem. Commun.* 1989, 1533-1535.
- (112) Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* 1988, 110, 584-589.
- (113) Kim, M.-J.; Hennen, W. J.; Sweers, H. M.; Wong, C.-H. *J. Am. Chem. Soc.* 1988, 110, 6481.
- (114) Adelhorst, K.; Björkling, F.; Godtfredsen, S. E.; Kirk, O. *Synthesis* 1990, 112-115.
- (115) Ciuffreda, P.; Colombo, D.; Ronchetti, T.; Toma, L. *J. Org. Chem.* 1990, 55, 4187-4190.
- (116) Colombo, D.; Ronchetti, F.; Toma, L. *Tetrahedron* 1991, 47, 103-110.
- (117) Look, G. C.; Ichikawa, Y.; Shen, G.-J.; Cheng, P.-W.; Wong, C.-H. *J. Org. Chem.* 1993, 58, 4326-4330.
- (118) Kefurt, K.; Kefurtova, Z.; Jary, J.; Horakova, I.; Marek, M. *Carbohydr. Res.* 1992, 223, 137-145.
- (119) Kloosterman, M.; Schoemaker, H. E.; Kloosterman-Castro, E. N.; Meijer, E. M. *Book of Abstracts: XXth European Carbohydrate Symposium*, Darmstadt, 1987, Lichtenthaler, F. W., Nett, K. K., Eds.; Frankfurt, 1987; p D-17.
- (120) (a) Wong, C.-H.; Chen, S.-T.; Hennen, W.-J. Bibbs, J. A.; Wang, Y.-F.; Liu, J. L.-C.; Pantoliano, M. W.; Whitlow, M.; Bryan, P. N. *J. Am. Chem. Soc.* 1990, 112, 945-953. (b) Zhong, Z.; Liu, J. L.-C.; Dinterman, L. M.; Finkelman, M. A. J.; Mueller, W. T.; Rollence, M. L.; Whitlow, M.; Wong, C.-H. *J. Am. Chem. Soc.* 1991, 113, 683-684.
- (121) Nicotra, F.; Riva, S.; Secundo, F.; Zuchelli, L. *Tetrahedron Lett.* 1989, 30, 1703-1704.
- (122) Carrea, G.; Riva, S.; Secundo, F. *J. Chem. Soc., Perkin Trans. 1* 1989, 1057-1061.
- (123) Cai, S.; Hakomori, S.; Toyokuni, T. *J. Org. Chem.* 1992, 57, 3431-3437.
- (124) Danielli, B.; De Bellis, P.; Carrea, G.; Riva, S. *Helv. Chim. Acta* 1990, 73, 1837-1844.
- (125) Kloosterman, M.; Weijnen, J. G. J.; De Vries, N. K.; Mentech, J.; Caron, I.; Descotes, G.; Schoemaker, H. E.; Meijer, E. M. *J. Carbohydr. Chem.* 1989, 8, 693-704.
- (126) Chang, K.-Y.; Wu, S.-H.; Wang, K. T. *Carbohydr. Res.* 1991, 222, 121-129.
- (127) Chang, K.-Y.; Wu, S.-H.; Wang, K. T. *J. Carbohydr. Chem.* 1991, 10, 251-261.
- (128) Ong, G.-T.; Wu, S.-H.; Wang, K. T. *Bioorg. Med. Chem. Lett.* 1992, 2, 161-164.
- (129) Sachdev, H. S.; Starkovsky, N. A. *Tetrahedron Lett.* 1969, 9, 733-736.
- (130) Taunton-Rigby, A. *J. Org. Chem.* 1973, 38, 977-985.
- (131) Moris, F.; Gotor, V. *J. Org. Chem.* 1992, 57, 2490-2492.
- (132) Gotor, V.; Moris, F. *Synthesis* 1992, 626-628.
- (133) (a) Moris, F.; Gotor, V. *Tetrahedron* 1992, 45, 9869-9876. (b) Moris, F.; Gotor, V. *Tetrahedron* 1993, 49, 10089-10098. (c) Moris, F.; Gotor, V. *J. Org. Chem.* 1993, 58, 653-660. (d) Garcia-Alles, L. F.; Moris, F.; Gotor, V. *Tetrahedron Lett.* 1993, 34, 6337-6338.
- (134) Uemura, A.; Nozaki, K.; Yamashita, J.-I.; Yasumoto, M. *Tetrahedron Lett.* 1989, 30, 3817-3818.
- (135) Nozaki, K.; Uemura, A.; Yamashita, J.-I.; Yasumoto, M. *Tetrahedron Lett.* 1990, 31, 7327-7328.
- (136) Uemura, A.; Nozaki, K.; Yamashita, J.-I.; Yasumoto, M. *Tetrahedron Lett.* 1989, 30, 3819-3820.
- (137) Danielli, B.; De Bellis, P.; Carrea, G.; Riva, S. *Gazz. Chim. Ital.* 1991, 121, 123-125.
- (138) Danielli, B.; De Bellis, P.; Carrea, G.; Riva, S. *Heterocycles* 1989, 29, 2061-2064.
- (139) Lehmann, H.; Miersch, O.; Schüttle, H. R. *Z. Chem.* 1975, 15, 443.
- (140) Schneider, G.; Miersch, O.; Liebisch, H.-W. *Tetrahedron Lett.* 1977, 405-406.

- (141) Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 3093-3096.
- (142) Margolin, A. L.; Delinck, D. L.; Whalon, M. R. *J. Am. Chem. Soc.* **1990**, *112*, 2849-2854.
- (143) Gardossi, L.; Bianchi, D.; Klivanov, A. M. *J. Am. Chem. Soc.* **1991**, *113*, 6328-6329.
- (144) Riva, S.; Klivanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 3291-3295.
- (145) Riva, S.; Bovara, R.; Ottolina, G.; Secundo, F.; Carrea, G. *J. Org. Chem.* **1989**, *54*, 3161-3164.
- (146) Njar, V. C. O.; Caspi, E. *Tetrahedron Lett.* **1987**, *28*, 6549-6552.
- (147) Parmar, V. S.; Sharma, N. K.; Bisht, K. S.; Sinha, R.; Taneja, P. *Pure Appl. Chem.* **1992**, *64*, 1135-1139.
- (148) Natoli, M.; Nicolosi, G.; Piattelli, M. *Tetrahedron Lett.* **1990**, *31*, 7371-7374.
- (149) Natoli, M.; Nicolosi, G.; Piattelli, M. *J. Org. Chem.* **1992**, *57*, 5776-5778.
- (150) Ramaswamy, S.; Morgan, B.; Ochischilager, A. C. *Tetrahedron Lett.* **1990**, *31*, 3405-3408.
- (151) Parmar, V. S.; Sinha, R.; Bisht, K. S.; Gupta, S.; Prasad, A. K.; Taneja, P. *Tetrahedron* **1993**, *49*, 4107-4116.
- (152) Steffen, B.; Ziemann, A.; Lang, S.; Wagner, F. *Biotechnol. Lett.* **1992**, *14*, 773-778.
- (153) Schuch, R.; Mukherjee, K. D. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 332-336.
- (154) Akoh, C. C.; Copper, C.; Nwuso, C. V. *J. Am. Oil Chem. Soc.* **1992**, *69*, 257-260.
- (155) Regen, S. L.; Singh, A.; Oehme, G.; Singh, M. *J. Am. Chem. Soc.* **1982**, *104*, 791-795.
- (156) Lin, G.; Wu, F.-C.; Liu, S.-H. *Tetrahedron Lett.* **1993**, *34*, 1959-1962.
- (157) Morimoto, T.; Murakami, N.; Nagatsu, A.; Sakakibara, J. *Tetrahedron Lett.* **1993**, *34*, 2487-2490.
- (158) Murakami, N.; Imamura, H.; Morimoto, T.; Ueda, T.; Nagai, S.; Sakakibara, J.; Yamada, N. *Tetrahedron Lett.* **1991**, *32*, 1331-1334.
- (159) Kitagawa, I.; Hayashi, K.; Kobayashi, M. *Chem. Pharm. Bull.* **1989**, *37*, 849-855.
- (160) Danieli, B.; De Bellis, P. *Helv. Chim. Acta* **1992**, *75*, 1297-1304.