P 82 Semi-synthesis of Peptides and Proteins

PROTEIN PHOSPHORYLATION VIA AMINO GROUP SUBSTITUTION: SEMISYNTHESIS OF PHOSPHORYLATED INSULINS.

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Protein phosphorylation plays a predominant role in biochemistry, and phospho peptides gain increasing interest. However, the introduction of phospho groups into larger peptides by global phosphorylation of OH groups is unsatisfactory. For example, it was not possible for us to isolate homogeneous derivatives from the complex mixtures obtained upon reaction of insulin with phosphorylating agents.

We have now studied the directed incorporation of phospho groups into insulin as a small model protein by coupling of phosphorylated amino acids to partially N-protected intermediates. Acylation (DCC/HOBt) of A1,B29-Msc2-insulin with Fmoc-X(POBzlOH) led to elongation at the B chain N-terminus, of A1,B1-Msc2-insulin to side chain substitution of B29-Lys (X = Tyr or Thr). Msc- and Fmoc groups were removed simultaneously with 10% aqueous pyridine, and subsequent debenzylation was effected with 95% TFA (3h). Preparative rp-HPLC gave the 8 analogues with free or monobenzylated phospho group and purity of >95%, which were characterized by MALDI-TOF-MS, amino acid analysis and electrophoresis: $Tyr(PO(OBzl)OH)^{B0}$-insulin (**1**), $Tyr(PO(OH)_2)^{B0}$- (**2**), $Thr(PO(OBzl)OH)^{B0}$-, $Thr(PO(OH)_2)^{B0}$-, $^{N_\varepsilon B29}$Tyr(PO(OBzl)OH)-Insulin (**5**), $^{N_\varepsilon B29}Tyr(PO(OH)_2)$-, $^{N_\varepsilon B29}$Thr(PO(OBzl)OH)-, $^{N_\varepsilon B29}Thr(PO(OH)_2)$-Insulin (**8**). Binding to insulin receptors in cult. IM-9 lymphocytes was 85% for **1**, 18% for **5**, and 30 – 70% for the others. CD spectra in the near and far uv showed only little structural effect of the phospho groups, as exemplified with **2** and **8**. We conclude that incorporation of such ready-made synthons is an effective method for site-directed phosphorylation of polypeptides and small proteins. We thank: A. Wollmer, M. Casaretto and S. Rütten. Supported by DFG (BR 651/14-1).

P 83

Enzymatic Peptide Synthesis

P 84

ENZYMATIC PEPTIDE SYNTHESIS CATALYZED BY PVA-CRYOGEL-ATTACHED SUBTILISIN 72 IN MEDIA WITH HIGH CONTENT OF ORGANIC SOLVENTS.

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The presence of organic solvents in media for biocatalytic reactions provides for numerous advanteges, especially when peptide synthesis is considered. The serious drawback of such systems is connected with enzyme inactivation at comparatively low concentration of organic solvents.

The model reaction Z-Ala-Ala-Leu-OCH_3 + Phe-pNA ——→ Z-Ala-Ala-Leu-Phe-pNA + CH_3OH catalyzed by PVA-cryogel-coupled subtilisin 72 in the mixtures CH_3CN/DMF with 60, 80, and 95% DMF was studied (for the PVA-cryogel immobilized enzyme preparation see [1]). The dependence of the yield of Z-Ala-Ala-Leu-Phe-pNA on the enzyme concentration in the mixture 80%DMF/20%CH3CN was investigated in the range 3.7 - 38 mkM. The reaction was carried out with equimolar amounts of amino- and acylating components and at [E]/[S] molar ratio of 1:200. Reactions in media with different CH_3CN/DMF ratio were carried out consequently with one and the same portion of immobilized subtilisin. After 2h the product yield was 85, 76 and 21% in systems with 60, 80, and 95% DMF, respectively. After this, enzyme preparation was placed again into the system with 60%DMF, which resulted in product yields of 41% and 85% after 2 h and 24 h reaction time, respectively. Thus, immobilized subtilisin retains high catalytic activity after several reaction cycles.

It was found that Z-Ala-Ala-Leu-OH is a good substrate of PVA-cryogel-attached subtilisin and the product yield being nearly the same as in the case of Z-Ala-Ala-Leu-OCH_3. Thus, the immobilized enzyme is capable to transforming an acyl donor with free carboxylic group. In the case of N-acylated tripeptides containing non-protected C-terminal basic and acidic amino acid residues (Z-Ala-Ala-Lys-OH and Z-Ala-Ala-Glu-OH) the reaction yield was of 82 and 86%, respectively.

1. Lozinsky V.I., Plieva F.M., Zubov A.L., Biotekhnologiya 1-2, 32 (1995) /in Russian/.

SYNTHESIS OF THE CHOLECYSTOKININ PEPTIDE CCK4 EXCLUSIVELY BY ENZYMATIC METHODS

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The aim of this investigation is to demonstrate the possibility of synthesising peptides exclusively by enzymatic methods in reasonable yields. This includes the enzymatic cleavage of the protection groups. If possible free enzymes are to be replaced by immobilised enzymes. The advantage of immobilised enzymes lies in their repeated use. The even more important advantage is the easy removal of the enzyme after the reaction, this being mandatory for therapeutical applications. As test peptide we choose CCK4.

The synthesis of the CCK4 tetrapeptide Trp-Met-Asp-Phe-NH_2 is achieved with three proteases and penicillin G amidase (PGA) in four enzymatic reaction steps. The starting materials are Phac-Trp, Met-OEt•HCl, Asp(OMe)-OMe•HCl and Phe-NH_2•HCl. All of them can be synthesised easily.

In the first step Phac-Trp (Phac = phenylacetyl) is coupled with Met-OEt•HCl in a yield of 60% or better with free and immobilised α-chymotrypsin. In a single reaction, the peptide bond is formed and the ester is hydrolysed. The resulting dipeptide with the free carboxylic group, Phac-Trp-Met-OH, is then coupled directly with Asp(OMe)-OMe•HCl in 66% yield with papain. Once again, the peptide bond is obtained and the α-ester is hydrolysed while the ß-ester is conserved. This is important since with thermolysin (TLN) the peptide bond with Phe-NH_2 can be obtained in high yield only in the presence of the ß-ester.

Under carefully controlled conditions almost no byproducts are observed. After prolonged reaction in the last coupling step however, Phac-Trp-Phe-NH_2 is formed slowly. Apparently TLN attacks the Trp-Met bond. The Phac-Trp then reacts with Phe-NH_2. This product has the lowest solubility and would finally be the end product in this thermodynamically controlled reaction. However, if the reaction is stopped in time this side reaction can be avoided completely.

The peptides are purified after each coupling step by preparative reversed phase chromatography under isocratic conditions with aqueous MeOH, buffered with 0.005 M ammonium acetate. The ß-ester of the aspartic acid is removed under elevated pH. With immobilised PGA the Phac group is removed easily.

The correct molecular mass of all peptides is verified by FAB-MS or FD-MS.

P 85

ENZYMATIC PEPTIDE SYNTHESIS IN ALCOHOLS CATALYZED BY SUBTILISIN IN THE COMPLEX WITH SODIUM DODECYL SULFATE

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The non-covalent complex of subtilisin 72 with sodium dodecyl sulfate (SDS) has been shown to catalyze numerous peptide bond coupling reactions. These were carried out in ethanol and isopropanol with an addition of 30% either DMF or DMSO as a cosolvents. Tetrapeptides Z-Ala-Ala-P_1-P_1'-pNA, where P_1=Ala, Leu, Phe, Trp, Tyr, Met, Phe(NO_2), Glu(OCH_3), Glu, Lys, Arg, His; P_1'= Ala, Leu, Phe, Ile, Val, Glu, Arg have been obtained with a good yields after 2 h when [S]/[E] = 5000:1. The activation of an acyl donor was demonstrated not to be much essential for the peptide bond forming which allowed to use peptides containing C-terminal free carboxyl groups as the acylating components.Using SDS-subtilisin as a catalyst, the feasibility of peptide segments coupling performed either in solution or on solid-phase support was shown. A series of varies penta- and hexapeptides including chromogenic and fluorogenic substrates for proteases was synthesized in ethanol. Z- or o-aminobenzoyl - protected di- and tripeptides were used as acyl donors, while di- and tripeptides containing C-terminal p-nitroanilide or N-2,4-dinitrophenylethylendiamine moietes were chosen as an amino components. SDS-subtilisin catalyzed coupling between peptides-spacers attached to aminosilichrom and N-protected peptide methyl esters in ethanol/DMSO mixture. Tree-step couplings of peptide segments by the 3+3+3 scheme were accomplished. The dependence of the reaction efficiency on the length and structure of spacers peptides, spacer density, reaction time, as well as concentrations of starting components and SDS-subtilisin was studied.

P 86

INFLUENCE OF REACTION CONDITIONS ON PEPTIDE BOND FORMATION CATALYZED BY LIPASES

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Lipases have been widely used in organic reactions involving esters[1]. Nevertheless, only a few researchers have tried to employ these enzymes for peptide bond formation through aminolysis of α-esterified amino acids[2]. As we are interested in preparing peptides by using such an enzymatic approach, we studied the influence of reaction conditions on the efficiency of the coupling between Ac-Tyr-OEt and Gly-NH_2. Purified *Candida cylindracea* lipase (CCL), purified porcine pancreatic lipase (pPPL) and the crude form of PPL (cPPL) were tested. The reactions were performed at 22 or 37°C under shaking at 300 rpm in a monophasic (Tris-HCl buffer, pH 8.0) or biphasic (n-hexane/buffer, 80/20, v:v) system, using 0.05 M Ac-Tyr-OEt, 0.07 or 0.5 M Gly-NH_2.HCl (neutralized or not) and 50 mg/mL of enzyme. The dipeptide formation was detected by RP-HPLC. Its identity was confirmed by isolation followed by characterization (amino acid analysis and electrospray mass spectrometry) and, also, by coelution with the synthetic standard obtained by the chemical method. A coupling yield of 92% was obtained in a 5 minute reaction carried-out at 37°C in the biphasic system using 0.05 M Ac-Tyr-OEt, 0.50 M Gly-NH_2 and pPPL. Temperature diminution or change to cPPL did not affect the reaction yield. On the other hand, the use of monophasic system at 22°C, a decrease of Gly-NH_2 concentration to 0.07 M and the employment of Gly-NH_2.HCl instead of Gly-NH_2 led to reaction yields of 67, 57 and 30%, respectively. All the reactions conducted in the presence of CCL gave very low amounts of the desired Ac-Tyr-Gly-NH_2 (~26%) even after 24h of reaction. These results indicate that: i) it is feasible to use a very cheap form of lipase (cPPL) as catalyst in peptide bond formation; ii) for our purposes, purified CCL is not as good as PPL; iii) as expected, some reaction condition parameters have a major influence on the coupling efficiency. Indeed, the amount of the amine component and the type of solvent system seem to be crucial. Some of our observations may be related to the well known lipase interface activation. [*Supported by FAPESP and CNPq*]

[1] Faber, K., *Biotransformations in Organic Chemistry*, 3rd. Edition, 1997; [2] So, J-E et al, *Enzyme Microb.Technol.*, **23**, 211, 1998

Posters: topic A4

P 87

SEMI-ENZYMATIC SYNTHESIS OF PEPTIDE ALDEHYDE INHIBITOR FOR GLU,ASP-SPECIFIC SERINE PROTEASE.

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Glutamyl-endopeptidases belong to a rather new family of the serine proteinases and possess narrow substrate specificity. They split the peptide bonds forming by carboxyl groups of dicarbonic amino acids, moreover cleavage of Glu-containing peptides is 100 time faster than peptides with Asp residues. There is no satisfactory explanation of this extremely strict specificity of glutamyl endopeptidases and amino acid residues, which are responsible for binding of substrate ω-carboxyl group, are not still determined. Therefore investigation of the interaction between enzyme and peptide inhibitor, for example peptide aldehyde, could be useful for understanding of their active center structures and catalysis mechanism.
Peptide aldehydes with Glual (e.g. Glual – glutamilal) as its C-terminal moiety was prepared via enzymatic acylation of preliminary obtained by chemical route glutamic acid aldehyde semicarbazone (e.g. Glual=N-NH-CO-NH_2 – glutamilal semicarbazone). Two-fold excess of N-acyltripeptide methyl ester was used as a carboxyl component.

$$Z\text{-Ala-Ala-Leu-OCH}_3 + \text{H-Glual=N-NH-CO-NH}_2 \xrightarrow{\text{subtilisin}}$$

Z-Ala-Ala-Leu-Glu=N-NH-CO-NH_2

The reaction was performed in organic solvent with low water content in presence of subtilisin 72 – serine proteinase from B. subtilis, distributed over a macroporous silica support surface. The structure of an amino aldehyde semicarbazone resembles that of a peptide one that improves its binding with subtilisin S1'-subsite and the conversion of the amino component to the peptide product was nearly quantitative. After removing of protective group peptide derivative with free aldehyde function that gave silvered mirror reaction was obtained. The inhibition parameters for this compound were determined in the hydrolysis reactions of corresponding chromogenic substrate for the enzymes from *Bacillus intermedius* and *Bacillus licheniformis.*

P 88

PEPTIDE SYNTHESIS MEDIATED BY MICROBIAL PROTEASES

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Although protease-catalyzed peptide synthesis possesses many advantages over chemical methods, a narrow substrate specificity and the secondary hydrolysis of a growing peptide are counted as major drawbacks. We have recently reported on the broadening of the α-chymotrypsin's substrate specificity by using such activated esters as the carbamoylmethyl ester as an acyl donor in the kinetically controlled peptide bond formation [1]. Microbial proteases from a variety of sources are available, but so far they have rarely been employed for synthetic purposes. Accordingly, we have intended to utilize effectively for peptide synthesis the catalytic activities of some microbial (e.g., *Bacillus subtilis* and *Aspergillus oryzae*) proteases which we used previously for the resolution of non-protein amino acids [2]. In the *B. subtilis* protease-catalyzed couplings of *N*-protected amino acid esters (Z-Xaa-OR) with amino acid amides (Xbb-NH_2) in acetonitrile with low water content, the effectiveness of the carbamoylmethyl ester as the acyl donor was once again ascertained in many cases. Furthermore, when Xaa = L-Ala and Xbb = L-Phe, for example, the gradually increasing by-product (Z-Xaa-OH) from the carbamoylmethyl ester turned to a decrease after several hours, suggesting the additional formation of the peptide product via Z-Xaa-OH. In fact, the independently performed coupling of Z-Xaa-OH with Xbb-NH_2 proceeded more rapidly than that of the corresponding ester substrate in some cases.

1) T. Miyazawa et al., *Chem. Lett.*, **1999**, 1013.
2) T. Miyazawa et al., *Tetrahedron: Asymmetry*, **8**, 367 (1997).

P 89

SUBTILISIN CATALYZED SYNTHESIS OF THROMBIN AND PLASMIN PEPTIDE INHIBITORS.

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Thrombin and plasmin are serine proteases of a key role in blood coagulation cascade. Specific inhibitors of these enzymes can be an efficient for treatment of such diseases as thrombosis and haemorrhage. There is a number of peptide derivatives known to be specific inhibitors of serine and thiol proteases. The most important of them are the peptide aldehydes. We proposed two enzymatic methods for preparing hydrophilic peptide aldehydes (peptidyl argininal and peptidyl lysinal). These approach allowed to avoid the problems of the functional groups protection and deprotection. The first route consists in enzymatic acylation of amino alcohols (Argol, Lysol) with N-protected peptide esters and subsequent mild oxidation of the product by anhydrous DMSO and 3-fold excess of acetic anhydride. The second one is enzymatic acylation of aldehyde semicarbazones (Argal-Sem, Lysal-Sem) preliminary obtained by chemical route amino. Subtilisin 72 (an enzyme closely related to subtilisin Carlsberg) distributed over macroporous silica was used as a catalyst in both cases. The reactions were performed in a mixture of organic solvents with a low water content. A series of the peptide derivatives was obtained by these methods (Z-Ala-Ala-Argol, Z-Ala-Phe-Lysol, For-Ala-Phe-Lysol, Z-Ala-Ala-Argal-Sem, Z-Ala-Ala-Lysal-Sem, For-Ala-Phe-Lysal-Sem). The yield of the products in both enzymatic reactions as 70-99%.

The inhibition parameters for dipeptidyl argininal and dipeptidyl lysinal obtained by one of these routes were determined in the reaction hydrolysis of chromogenic substrates by thrombin and plasmin. Z-Ala-Ala-Argal shows an inhibition of thrombin stronger than plasmin. Whereas For-Ala-Phe-Lysal is a specific plasmin inhibitor and it does not affect on thrombin activity. Both peptide derivatives are stable and do not loose their efficiency toward target enzymes in blood plasma.

P 90

ENGINEERING OF TRYPSIN FOR SUBSTRATE MIMETICS-MEDIATED PEPTIDE SYNTHESIS

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The use of substrate mimetics as acyl donor components in protease-catalyzed peptide synthesis is a promising method to overcome the limitation given by the original specificity of these enzymes. Recent papers have been demonstrated that on the basis of this novel strategy, a wide range of nonspecific acyl moieties comprising noncoded and even non-amino acid-derived acyl residues can be coupled by the native proteases.[1] On the other side, the universal use of this beneficial approach is seriously restricted by the permanent risk of unwanted proteolytic cleavages caused by the intrinsic amidase activity of these enzymes. Thus, further efforts are essentially needed to broad the scope of substrate mimetics for enzymatic synthesis.
The subject of this work is the use of site-directed mutagenesis for the design of new and improved trypsin variants which are catalytic active for the synthesis of peptide bonds, but generally inactive for their cleavage. Several acyl transfer experiments have indicated the outstanding functional importance of Asp 189 within trypsin for the specificity and especially for the native amidase activity of the enzyme. We found that in particular the exchange of this acidic residue with basic amino acid moieties like lysine, arginine, and histidine decreases the proteolytic activity of the enzyme to a significantly higher extent than the corresponding esterase activity which is required for synthesis. The combination of this mutation with a second amino acid exchange, i.e. Lys 60 of trypsin with Glu, finally leads to enzyme variants with a high activity toward esters of the type of substrate mimetics, but practically no activity for cleaving peptide bonds. As an important result, these trypsin variants catalyse substrate mimetics-mediated peptide ligations practically without proteolytic side reactions. Consequently, the utilization of this artificial enzyme species combined with the use of the substrate mimetics-strategy significantly broads the range of synthetic applications of proteases for selective ligations of peptides.

[1] see review: Bordusa, F. (2000). *Braz. J. Med. Biol. Res.*, in press.

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Posters: topic A4

P 91 Enzymatic Peptide Synthesis P 92

MECHANISM OF PSEUDOLYSIN-CATALYZED SYNTHESIS OF Z-ALA-PHE-NH_2

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We previously demonstrated that pseudolysin, a neutral metalloproteinase from thermolysin family, is a suitable catalyst for peptide bond formation through reverse proteolysis. In order to get an insight into the yield- and rate-controlling factors, we studied kinetics and mechanism of pseudolysin-catalysed synthesis. The condensation of Z-Ala-Phe-NH_2 from Z-Ala and Phe-NH_2, performed in a semi-organic homogeneous system, was chosen as a reaction model. For measuring the initial rates of the enzymatic reaction, we applied both conductumetric and chromatographic (HPLC) methods, before any product precipitation. We have first optimised synthesis and conductimetric experimental conditions.The highest velocities were obtained in the 7.0-7.5 range of pH. When kept below 0.15, ionic strength did not significantly alter the rate values. Kinetic studies were then performed for varied Z-Ala and Phe-NH_2 concentrations at different, fixed concentrations of the second substrate, at pH 7.3 in 35% MeOH and for a ionic strength value below 0.15. Graphical inspection and numerical analysis of initial-rate data allowed calculation of the kinetic parameters and proposition of a kinetic mechanism for pseudolysin. The values of kinetic constants were : 6.5 mM Z-Ala-Phe-NH_2 synthesised/min/µM pseudolysin (Vm), 70 mM (K_M of Z-Ala) and 180 mM (K_M of Phe-NH_2). In addition, the condensation reaction proceeded via a rapid-equilibrium random bireactant mechanism, as it was previously demonstrated for thermolysin, an other extensively studied metalloprotease.
A similar result was obtained when HPLC was used for monitoring velocities in a broader range of substrates (40-200 mM for Z-Ala and 50-1200 mM for Phe-NH_2).

S. Rival, J. Saulnier and J. Wallach (1999) Biocatalysis and Biotransformation, in press.

CHEMO-ENZYMATIC SYNTHESIS OF α-AMINOACIDS ACYLASES AND AMINO-OXYDASES

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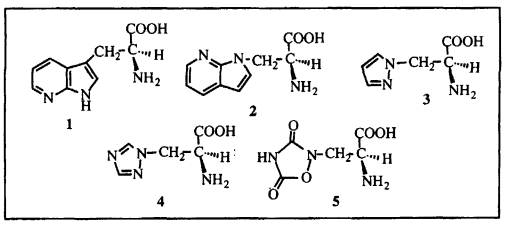
(S) 7-Azatryptophane, the fluorescent probe **1**, was prepared by enzymatic resolution (1) (2). In this piece of work we describe different ways to synthesize optically pure β-heterocyclic aminoacids and γ-hydroxyaminoacids: 7-azaindolylalanine **2**, pyrazolylalanine **3** and 1,2,4-triazolylalanine **4**, quisqualic acid **5** (3) and γ-hydroxyisoleucine isomers. We also describe a rapid and efficient synthesis of amino acid derivatives performed on solid support and applicable in combinatorial chemistry (4).



(1) Lecointe L., Rolland-Fulcrand V., Roumestant M.L., Viallefont Ph., Martinez J Tetrahedron Asym., 1998, 9, 1753-1758. (2) L. Pappalardo, J.M. Receveur, V. Rolland, Y. Vidal, M.L. Roumestant, Ph. Viallefont, 1999, ,Recent Res. Devel. Synth. Org. Chem., 2: 35-47. (3) Rolland V., Haroune N., Roumestant M.L., Martinez J. Tetrahedron: Asym. 2000, in preparation.. (4) Barbaste M., Rolland-Fulcrand V., Roumestant M.L., Viallefont Ph., Martinez J., Tetrahedron Lett. 1998, 39, 6287-6290.

P 93 P 94

OLIGOPEPTIDE SYNTHESIS USING INVERSE SUBSTRATES: SEGMENT CONDENSATION CATALYZED BY BOVINE, *STREPTOMYCES GRISEUS*, AND CHUM SALMON TRYPSINS

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Peptide synthesis by protease-catalyzed reverse reaction has been extensively studied with a variety of model oligopeptides. It is known that enzymatic peptide synthesis is more advantageous than chemical synthesis in many respects; it is highly stereoselective and racemization-free, and requires minimal side-chain protection. The most serious defect of the enzymatic method, however, is the restrictive substrate specificity. Thus, the application of proteases for peptide synthesis has been limited by the specificity of the enzymes.

Previously, we reported that inverse substrates such as *p*-amidino- and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes and allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site. The characteristic features of inverse substrates suggested that they are useful for enzymatic peptide synthesis. We demonstrated successful application of inverse substrates for trypsin-catalyzed coupling.

It was reported that trypsin from cold-adapted species have been shown to be more efficient catalysis than their mammalian counterparts. Thus, trypsin from cold-adapted species could be expected highly efficient for enzymatic peptide synthesis. Recently, we reported the isolation and properties of trypsin from Chum salmon (*Oncorhynchus keta*). Herein, we describe studies on the utility of inverse substrates for segment condensation by bovine trypsin in comparision to reactions catalyzed by *Streptomyces griseus* (SG) and chum salmom trypsins, which had not yet been exploited for this strategy.

The coupling reaction was carried out by incubating an acyl donor (inverse substrates = N^{α}-Boc-peptide *p*-guanidinophenyl esters (-OGp), 25 mM) with an acyl acceptor (peptide ester, 250 mM) and enzyme (250 µM) in a mixture of MOPS buffer (50 mM, pH 8.0) and DMSO (1:1) at 25 °C. The progress of the coupling reaction was monitored by HPLC. The emzymatic synthesis of N^{α}-Boc-Try-Gly-Gly-Phe-Leu-OR (R = methyl, *tert*-butyl and benzyl) was examined by segment condensation of N^{α}-Boc-Try-Gly-Gly-OGp and Phe-Leu-OR. All trypsins were effective for the segment condensation. Detail of this method with typical example will be discussed.

PROTEASE-CATALYZED SYNTHESIS OF α-FLUOROALKYL SUBSTITUTED PEPTIDES

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Many peptides are used as therapeutic agents. A major drawback of these substances is their rapid degradation by proteases and the lack of transport systems through cell membranes. The incorporation of α,α-disubstituted amino acids into strategic position of biological active peptides represents one possibility for increasing metabolic stability, improving lipophilicity and stabilization of secondary structures.[1] α-Fluoroalkyl substituted amino acids are a special class of α,α-disubstituted amino acids and play an important role for modification of peptides. The fluorine atom with its high electronegativity increases the polarity of neighbouring substituents and is capable of participating in hydrogen bondings.[2,3] Furthermore, stronger interactions between fluorine-containing substances and receptors or enzyme subsites occur and can be used in protease-catalyzed peptide synthesis.
The application of proteases especially for C-N ligations is an attractive alternative to the chemical methods, because the enzymatic formation of peptide bonds is highly regio- and stereospecific and, therefore, does not require large efforts to protect side chains of trifunctional amino acids.
Recently, the enzyme-catalyzed incorporation of α-fluoromethyl amino acids into the P_2, P_3 and P_2'-position (nomenclature according of Schechter and Berger) of peptide fragments has been successfully demonstrated.[4] Furthermore, the universal validity of the substrate mimetic concept in enzymatic peptide synthesis was expanded to the transfer of C-terminal α-fluoroalkyl substituted amino acids. Generally, each trifluoromethyl- and difluoromethyl amino acid 4-guanidinophenyl ester was able to serve as an acyl donor in analytical trypsin- and chymotrypsin-catalyzed peptide bond formations independently of its acyl moiety far away from the natural enzyme specificity.

1. J. Rizo, L. M. Giersch, *Ann. Rev. Biochem.* 1992, 61, 387.
2. N. Sewald, K. Burger, in V. P. Kukhar und V. A. Soloshonok (eds.), *Fluorine-containing Amino Acids, Synthesis and Properties*, John Wiley & Sons, Chichester, UK, 1995, 139-220.
3. B. Koksch, N. Sewald, H.-J. Hofmann, K. Burger, H.-D. Jakubke, *J. Peptide Sci.* 1997, 3, 157.
4. F. Bordusa, C. Dahl, H.-D. Jakubke, K. Burger, B. Koksch, *Tetrahedron Asymmetry*, 1999, 10, 303.
5. F. Bordusa, D. Ullmann, C. Elsner, H.-D. Jakubke, *Angew. Chem. Int. Ed. Engl.*, 1997, 36, 2473.
6. M. Thormann, S. Thust, H.-J. Hofmann, F. Bordusa, *Biochemistry* 1999, 38, 6056.

Posters: topic A4

P 95 Enzymatic Peptide Synthesis P 96

UNUSUAL REACTION OF PEPTIDE BOND FORMATION CATALYZED BY SUBTILISIN

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Serine proteinase subtilisin 72 is a convenient tool for peptide bond synthesis due to a rather broad specificity of its S'_1-subsite, which accepts differents amino component, not only L-amino acids. This endopeptidase is especially sensitive to the length of substrate. The best acylating component in subtilisin catalyzed reaction of peptide synthesis are acyl tripeptide esters.
We report here an unexpected ability of this enzyme to catalyze the peptide bond formation between free amino acid esters (including esters of non protein amino acids and amino acids not appropriate to the specificity requirements of S_1-subsite) and amino acid amides such as p-nitroanilides:

$$\text{H-Xaa-OCH}_3 + \text{H-Yaa-pNA} \xrightarrow{\text{subtilisin}} \text{H-Xaa-Yaa-pNA}$$

where Xaa = Leu, Phe, Val, Trp, Arg, Pro, Met, Ser, Ala, Glu, ε–AHA, D-Leu,
Yaa = Leu, Phe, Arg, pNA = p-nitroanilide

The reactions proceed in the organic media with a low water content. The catalyst was distributed over the surface of macroporous silica. Sometimes the excess of the amino acid ester allows enzymatic acylation of the dipeptide formed:

$$\text{H-Ser-OCH}_3 + \text{H-Ser-Phe-pNA} \xrightarrow{\text{subtilisin}} \text{H-Ser-Ser-Phe-pNA}$$

The products of these reactions are well distinguishable by HPLC analysis, their composition were confirmed by amino acid analysis. In water-organic mixtures the synthesis of peptides is handicapped by hydrolysis of the amino acid esters and peptides formed. In spite of this, some peptides could be obtained with a good yields:

$$\text{H-His-OCH}_3 + \text{Leu-pNA} \xrightarrow{\text{subtilisin}} \text{H-His-Leu-pNA}$$

In the absence of the amino component, the source amino acid ester is hydrolyzed completely rather quickly.

PROGRAMMING OF ENZYME SPECIFICITY BY SUBSTRATE MIMETICS: INVESTIGATIONS ON THE V8 PROTEASE

Nicole Wehofsky[a] and Frank Bordusa[b]
[a]University of Leipzig, Institute of Biochemistry, Germany; [b]Max-Planck-Society, Research Unit "Enzymology of Protein Folding", D-06120 Halle, Germany.

It has been demonstrated that peptide synthesis catalyzed by native proteases represents an attractive alternative to chemical synthetic methods because enzymatic reactions are regio- and stereospecific, free from racemisation, and only require a miminum of protecting group chemistry. Apart from this undisputed advantages, the universal approach is limited both by the restricted substrate specificity of proteases and the risk of undesired proteolysis of reactants and peptide products. Recently, we presented the application of the substrate mimetic concept to overcome this limitation.[1] Contrary to commonly used acyl donor esters, this special type of substrates characteristically bears an ester leaving group which interacts with the active site of proteases in a way that mimics specific side-chain functionalities.[2] As a result, peptide bond formations occur irreversibly and independently of primary specificity and, therefore, qualify the strategy of substrate mimetics as a powerful concept for the incorporation of nonspecific coded and noncoded amino acids and even non-amino acid-derived derivatives into peptides.[3] Based on the specificity determinants of Glu-specific endopeptidase from Staphylococcus aureus (V8 protease), in an empirical way a new type of substrate mimetics bearing anionic leaving groups was designed from simple structure-function relationship studies.[4,5] Finally, the succesful application of these artificial substrates for model segment condensations and selective N-terminal modifications of peptides could be demonstrated.

[1] Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. (1997). *Angew. Chem. Int. Ed. Engl. 36*, 2473.
[2] Thormann, M.; Thust, S.; Hofmann, H.-J.; Bordusa, F. (1999). *Biochemistry 38*, 6056.
[3] Günther, R.; Bordusa, F. (2000). *Chem. Eur. J. 6*, 463.
[4] Wehofsky, N.; Bordusa, F. (1999). *FEBS Letters 443*, 220.
[5] Wehofsky, N.; Wissmann, J.-D.; Alisch, M.; Bordusa, F. *Biochim. Biophys. Acta*, in press.

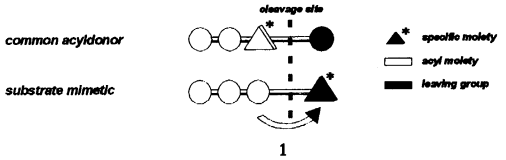
This work was supported by the Deutsche Forschungsgemeinschaft (INK 23) and the Fonds der Chemischen Industrie (Liebig-scholarship, F.B.).

P 97

NONCONVENTIONAL CN-LIGATION STRATEGY: PROGRAMMING OF ENZYME SPECIFICITY BY SUBSTRATE MIMETICS

Nicole Wehofsky[a], Kathrin Rall[a], Robert Günther[a], Vaclav Cerovsky[a], and Frank Bordusa[a,b]
[a]University of Leipzig, Institute of Biochemistry, Germany; [b]Max-Planck-Society, Research Unit "Enzymology of Protein Folding", D-06120 Halle, Germany

Due to the regio- and stereospecificity proteases considerably extend current methods of peptide synthesis by combining the advantages of biocatalysis with the flexibility of chemical approaches. Looking to the nature, the ideal enzyme for this purpose should be the ribosomal peptidyl transferase. Unfortunately, this enzyme activity is not suitable for simple practical use outside the ribosome. Therefore, the only alternative of practical importance for catalyzing peptide bond formation are proteases based on the principle of microscopic reversibility. Since the native function of this enzymes they do not act as perfect CN-ligases. As a consequence, several problems need to be considered, especially the limited enzyme specificity and the risk of undesired proteolysis of reactants and peptide products. The subject of the work presented, was to establish a novel strategy based on substrate mimetics (1) that allows an irreversible peptide bond formation which is independent of the primary specificity of proteases.[1-3]



1

[1] Bordusa, F., Ullmann, D., Elsner, C. & Jakubke, H.-D. (1997). *Angew. Chem. Int. Ed. Engl. 36*, 2473.
[2] Wehofsky, N. & Bordusa, F. (1999). *FEBS Lett. 443*, 220.
[3] Thormann, M., Thust, S., Hofmann, H.-J. & Bordusa, F. (1999). *Biochemistry 38*, 6056.

This work was supported by the Deutsche Forschungsgemeinschaft (INK 23) and the Fonds der Chemischen Industrie (Liebig-scholarship, F.B.).

P 98 Large-scale Peptide Synthesis P 99

ENZYMATIC PEPTIDE SYNTHESIS CATALYZED BY PVA-CRYOGEL-ATTACHED SUBTILISIN 72 IN MEDIA WITH HIGH CONTENT OF ORGANIC SOLVENTS.

Bacheva Anna V.[a], Plieva Fatima M.[b], Lysogorskaya Elena N.[a], Filippova Irina Yu.[a], Lozinsky Vladimir I.[b]
[a]Department of Chemistry, Moscow State University, [b]A.N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia

The presence of organic solvents in media for biocatalytic reactions provides for numerous advanteges, especially when peptide synthesis is considered. The serious drawback of such systems is connected with enzyme inactivation at comparatively low concentration of organic solvents.

The model reaction Z-Ala-Ala-Leu-OCH_3 + Phe-pNA ——→ Z-Ala-Ala-Leu-Phe-pNA + CH_3OH catalyzed by PVA-cryogel-coupled subtilisin 72 in the mixtures CH_3CN/DMF with 60, 80, and 95% DMF was studied (for the PVA-cryogel immobilized enzyme preparation see [1]). The dependence of the yield of Z-Ala-Ala-Leu-Phe-pNA on the enzyme concentration in the mixture 80%DMF/20%CH3CN was investigated in the range 3.7 - 38 mkM. The reaction was carried out with equimolar amounts of amino- and acylating components and at [E]/[S] molar ratio of 1:200. Reactions in media with different CH_3CN/DMF ratio were carried out consequently with one and the same portion of immobilized subtilisin. After 2h the product yield was 85, 76 and 21% in systems with 60, 80, and 95% DMF, respectively. After this, enzyme preparation was placed again into the system with 60%DMF, which resulted in product yields of 41% and 85% after 2 h and 24 h reaction time, respectively. Thus, immobilized subtilisin retains high catalytic activity after several reaction cycles.

It was found that Z-Ala-Ala-Leu-OH is a good substrate of PVA-cryogel-attached subtilisin and the product yield being nearly the same as in the case of Z-Ala-Ala-Leu-OCH_3. Thus, the immobilized enzyme is capable to transforming an acyl donor with free carboxylic group. In the case of N-acylated tripeptides containing non-protected C-terminal basic and acidic amino acid residues (Z-Ala-Ala-Lys-OH and Z-Ala-Ala-Glu-OH) the reaction yield was of 82 and 86%, respectively.

1. Lozinsky V.I., Plieva F.M., Zubov A.L., Biotekhnologiya 1-2, 32 (1995) /in Russian/.

ENZYMATIC PEPTIDE SYNTHESIS CATALYZED B'
CRYOGEL-ATTACHED SUBTILISIN 72 IN MEDIA '
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Bacheva Anna V.[a], Plieva Fatima M.[b], Lysogorskaya Elena N.[a], Filippova I
Lozinsky Vladimir I.[b]
[a]Department of Chemistry, Moscow State University, [b]A.N. Nesmeyanov I
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1. Lozinsky V.I., Plieva F.M., Zubov A.L., Biotekhnologiya 1-2, 32 (1995) /i
Russian/.

P 100

ENZYMATIC PEPTIDE SYNTHESIS CATALYZED BY PVA-CRYOGEL-ATTACHED SUBTILISIN 72 IN MEDIA WITH HIGH CONTENT OF ORGANIC SOLVENTS.

Bacheva Anna V.[a], Plieva Fatima M.[b], Lysogorskaya Elena N.[a], Filippova Irina Yu.[a], Lozinsky Vladimir I.[b]
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1. Lozinsky V.I., Plieva F.M., Zubov A.L., Biotekhnologiya 1-2, 32 (1995) /in Russian/.

Posters: topic A6

P 101

Chemistry of Amino-acids

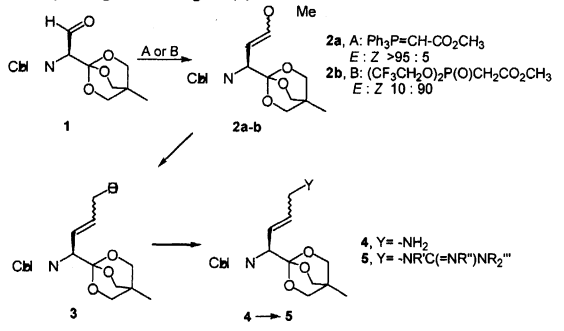
P 102

STEREOSELECTIVE SYNTHESES OF β,γ-UNSATURATED AMINO ACIDS

Maria C. Alcaro,[1] Anna M. Papini,[1] Mario Chelli,[1] Dan Fishlock,[2] Carolyn Atkins,[2] and Gilles A. Lajoie,[2] from [1]Dipartimento di Chimica Organica "Ugo Schiff" and CNR-CSCEA, Università degli Studi di Firenze, I-50121 Firenze, Italy; [2]Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1.

β,γ-Didehydroamino acids are interesting tools for a rational and systematic approach to peptide design, because they provide minimal but well defined, conformational constraints to the amino acid side chains. Once the optimal geometry is determined by structure-activity studies, it can serve as basis for the design of more elaborated peptide mimetics.

We report the synthesis of a series of β,γ-unsaturated α-amino acids bearing a functional group on the side chain, starting from the stereoisomers of 2-amino-5-hydroxypent-3-enoic acid (**3**) (β,γ-didehydro-δ-hydroxynorvaline). The different stereoisomers of **3** were obtained as enantiopure building-blocks from Cbz-(L or D)-Ser(ald)-OBO ester (**1**) [Lajoie, G.A., et al., *J. Am. Chem. Soc.*, 115 (1993) 5021-5030; *J. Org. Chem.*, 63 (1998) 3631-3646], *via* olefination and subsequent reduction of the side-chain methoxycarbonyl group of Cbz-(*E* or *Z*)-β,γ-didehydro-Glu(OMe)-OBO ester (**2**). Nucleophilic displacement of the hydroxy group of compound **3** led to (*E* or *Z*)-β,γ-didehydroornithine (**4**), which was converted to (*E* or *Z*)-β,γ-didehydroarginine analogues (**5**).



APPLICATION OF FMOC-DPR(IVDDE)-OH IN PEPTIDE SYNTHESIS

Jörg Beythien, Peter Schneeberger
Calbiochem-Novabiochem AG, Weidenmattweg 4, CH-4448 Läufelfingen, Switzerland

1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) [1] and 1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) [2] have been introduced as protecting groups for the amino groups of lysine, ornithine, diaminobutyric acid, and diaminopropionic acid (Dpr). The two protecting groups are quasi-orthogonal to the Fmoc group and can be removed using a solution of 2% hydrazine. Using multiple diamino acid derivatives with Dde protection in peptide synthesis, intra- and intermolecular migration of the Dde group has been shown [3]. The sterically hindered ivDde variant is considered more stable to piperidine and less prone to migrate. Nevertheless, migration of the ivDde protecting group in Dpr containing peptides does still occur [4]. Here we present our studies on coupling and cleavage conditions to minimize migration side products when using Fmoc-Dpr(ivDde)-OH in peptide synthesis.

[1] B.W. Bycroft, W.C. Chan, S.R. Chhabra, and N.D. Hone,(1993) J. Chem. Soc., Chem Commun., 778.
[2] S.R. Chhabra, B. Hothi, D.J. Evans, P.D. White, B.W. Bycroft, and W.C. Chan (1998) Tetrahedron Lett., **39**, 1603.
[3] K. Augustyns, W. Kraas, and G. Jung (1998) J. Peptide Res., **51**, 127.
[4] R.R. Wilhelm, A. Srinivasan, and M.A. Schmidt (1999), poster at the 16th APS, Minneapolis.

P 103

STEREOSELECTIVE SYNTHESIS OF *N*-BOC-PROTECTED-3,6-DISUBSTTUTED PIPERIDIN-2-ONES AND α,δ-DISUBSTITUTED δ- AMINO ACIDS

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[a]Laboratoire de Chimie Immunologique, UPR 9021 CNRS, IBMC, 15 rue Descartes, F-67000 Strasbourg, France. [b]ESA 7036 CNRS, LCM3B Université Henri Poincaré, BP239, F-54509 Vandoeuvre, France. [c]Neosystem, 7 rue de Boulogne, F-67100 Strasbourg, France.

The interest in ω-amino acids and ω-amino acid- containing peptides has increased enormously with the finding that short chain peptides consisting exclusively of optically active β- or γ-amino acids can form stable folded structures in solution and in the solid state [1, 2]. Recently, a few reports have highlighted the potential of higher amino acids, including δ-amino acids, for studying peptide folding [3] and for the construction of novel oligomers [4]. Optically active δ-amino acids with various substitution patterns and diverse side chain functionalities are thus of interest for further studies in this area. Additionally, α,δ-disubstituted δ-amino acids can serve as dipeptide mimetics in which the amide bond has been replaced by the nonhydrolysable ethylene unit ψ[CH_2CH_2].

Although the synthesis of α,δ-disubstituted δ-amino acids has been reported in the literature, no general, stereoselective synthetic route has been described so far. We report herein a general and stereoselective method for the preparation of these δ-amino acids which is amenable to the synthesis of all four possible diastereomers and which allows the introduction of various natural and unnatural sidechains at the α-position.

[1] Seebach, D.; Matthews, J. L. *Chem. Commun.* **1997**, 2015. [2] Hintermann, T.; Gademann, K.; jaun, B.; Seebach, D. *Helv. Chim. Acta* **1998**, *81*, 983. [3] Banerjee, A.; Pramanik, A.; Bhattacharjya, S.; Balaram, P. *Biopolymers*, **1996**, *39*, 769. [4] Szabo, L.; Smith, B. L.; McReynolds, K. D.; Parrill, A. B.; Morris, E. R.; Gervay, J. *J. Org. Chem.* **1998**, *63*, 1074.

P 104

SYNTHESIS OF α-AMINOPHOSPHINIC COMPOUNDS AS NEW DUAL INHIBITORS OF NEPRILYSIN AND AMINOPEPTIDASE N

H. CHEN[a], F. NOBLE[a], A. YIOTAKIS[b]; A. MAKARITIS[b]; P. GEORGE[c]; B. P. ROQUES[a] and M. C. FOURNIE-ZALUSKI[a]
aDépartement de Pharmacochimie Moléculaire et Structurale U266 INSERM-URA D1500 CNRS UFR des Sciences Pharmaceutiques et Biologiques 4, avenue de l'Observatoire.,75270 PARIS Cedex 06, France.
bDepartment of Chemistry, Laboratory of Organic Chemistry, University of Athens, Panepistimiopolis Zografou, 15771, Athens, Greece.
cSanofi-Synthelabo, 31, Avenue Paul Vaillant Couturier 92220 Bagneux, France.

The development of dual inhibitors of the two zinc metalloenzymes, neprilysin (neutral endopeptidase, NEP, EC, 3.4.24.11) and aminopeptidase N (APN, EC, 3.4.11.2) involved in the inactivation of the opioid peptides enkephalins, represent an attractive physiological approach in the search for new analgesics devoid of the major drawbacks of morphine1. NEP and APN belong to the same family of zinc metallopeptidases with the consensus sequence HEXXH, providing the possibility of designing a single molecule capable to efficiently block the active site of both enzymes. With this aim, we have synthesized a-aminophosphinic compounds corresponding to the general formula:
H3N+CH(R1)P(O)(OH)CH2CH(R2)CONHCH(R3)COO- able to act as transition state analogs, to interact with the S1, S'1 and S'2 subsites of the two enzymes and to fulfil the requirements for either a selective recognition of APN2 or a dual inhibition of both APN and NEP3. Selection of the R1, R2 and R3 residues for optimal recognition of these enzymes, led to the first dual competitive inhibitors with Ki values in the
nanomolar range for NEP and APN. These compounds induce potent antinociceptive responses after intracerebroventricular administration in mice (hot plate test), and several of them were shown to be, at least, ten times more potent than the previously described dual inhibitors. To increase the bioavailability of the most active inhibitors, prodrugs were developped, leading to compounds inducing long, lastingantinociceptive responses after i.v., i.p. and intranasal administrations.

References: (1) Roques, B. P.; et al, Pharmacol. Rev. 1993, 45, 87-146. (2) Chen, H.; et al, Bioorg. Med. Chem. Lett., 1999, 9, 1511-1516. (3) a, Chen, H.; et al, Proc. Natl. Acad. Sci. USA , 95, 12028-12033 (1998); b, Chen, H.; et al,, J. Med. Chem., (in press).

Posters: topic A6

P 105 — Chemistry of Amino-acids — P 106

C-TERMINAL AMIDE BOND STABILITY OF PEPTIDES CONTAINING $C^{\alpha,\alpha}$-DISUBSTITUTED GLYCINES

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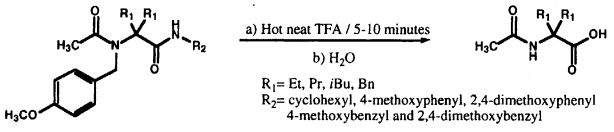
We have since long been interested in the synthesis of $C^{\alpha,\alpha}$-disubstituted glycines *via* a Ugi-Passerini Four Component Condensation Reaction [1]. Recently we have improved this method of synthesis by using 4-methoxybenzyl amine as the amine component of the reaction, which allowed an important simplification in the subsequent deprotection of the product.
During cleavage of the 4-methoxybenzyl group with hot neat TFA, we detected that not only this group was removed but also the C-terminal amide bond was cleaved [2]. This result was independent of the nature of both the amino acid residue generated and the *N*-substituent at the C-terminal amide group [3].
These results prompted us to a thorough study of the stability of this amide bond. For R_1=Et, Pr and *i*Bu, when HCl or TFA (either neat or 2% in acetonitrile) was used at room temperature, only the C-terminal amide bond was cleaved, while removal of the 4-methoxybenzyl group required neat TFA in much longer reactions. In the case of R_1= Bn, no selectivity was observed in neat TFA, as in this case the rate of cleavage of the amide bond was similar to that of the *N*-alkyl group [4].
We wish now to present the preliminary results of our investigation on the stability of the C-terminal amide bond in several peptide derivatives and also on the application of these results to peptide synthesis.



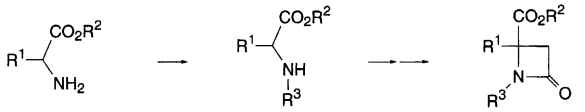
R_1= Et, Pr, *i*Bu, Bn
R_2= cyclohexyl, 4-methoxyphenyl, 2,4-dimethoxyphenyl 4-methoxybenzyl and 2,4-dimethoxybenzyl

[1] Maia, H.L.S., Ridge, B., Rydon, H.N., *J. Chem. Soc. Perkin Trans. I*, (1973), 98.
[2] a) Spencer, J.R., Delaet, N.G.J., Toy-Palmer, A., Antonenko, V.V., Goodman, M., *J. Org. Chem.*, (1993), **58**, 1635. b) Keating, T.A., Armstrong, R.W., *J. Org. Chem.*, (1998), **63**, 867.
[3] Results presented at the 16th APS, P022, Minneapolis, Minnesota, USA, July, 1999.
[4] Results presented at the VII EPI, oral presentation I-2, Valencia, Spain, February, 2000.

NEW CONFORMATIONALLY RESTRICTED AMINO ACID DERIVATIVES. SYNTHESIS OF 1,4,4-TRISUBSTITUTED AZETIDIN-2-ONES

G. Gerona-Navarro, M.A. Bonache, R. Herranz, R. González-Muñiz, and M. T. García-López
Instituto de Química Médica (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.

The incorporation of conformationally constrained amino acid derivatives into peptide sequences is a strategy widely used for the optimization of peptide ligand-receptor interactions. Therefore, methods giving access to new conformationally restricted non-proteinogenic amino acids, from easily available starting materials, could prove to be of a great value. In this sense, we have developed, and described here, a three-step synthetic route for the preparation of 1,4,4-trisubstituted azetidin-2-one derivatives from commercially available amino acid alkyl esters. These azetidin-2-one derivatives, combining C_α alkylation with N-C_α cyclization, are able to restrict the ϕ torsion angle of the corresponding amino acid close to 70 or -70°, depending on the absolute configuration at C-4.



R^1 = H, CH_3, $CH_2CH(CH_3)_2$, $(CH_2)_2CO_2{}^tBu$, CH_2Ph, CH_2In
R^2 = Me, ^tBu
R^3 = CH_2Ph, $CH_2Ph(4\text{-}OMe)$, H

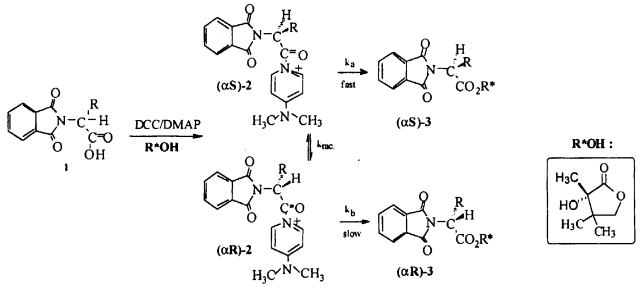
The key step of this synthetic route, involving the formation of the azetidin-2-one C_3-C_4 bond, was performed by intramolecular Cα-alkylation of the corresponding *N*-benzyl-*N*-chloroacetyl amino acid derivative. The scope and limitations of this new procedure towards 1,4,4-trisubstituted azetidin-2-one derivatives, along with the *N*-deprotection reactions, will be discussed in detail.

P 107 — P 108

DYNAMIC KINETIC RESOLUTION OF RACEMIC N-PHTALYL AMINO ACIDS USING (S)-α-METHYLPANTOLACTONE AS A NOVEL CHIRAL AUXILIARY

Christèle Glot, Monique Calmes, Thibaut Michel, Marc Rolland, Jean Martinez, Laboratoire des Aminoacides, Peptides et Protéines, UMR 5810 CNRS-UM1-UM2, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.

A novel type of dynamic kinetic resolution of racemic N-phthalyl amino acids by stereoselective esterification was examined using (S)-α-methylpantolactone as a chiral auxiliary. With one equivalent each of DCC and 4-dimethylaminopyridine (DMAP), this reaction allowed a dynamic kinetic resolution process due to the propensity of the corresponding acyl (4-dimethylamino)pyridinium salt to racemize.



This method provides a facile access to optically active α amino acids and can be applied to other α-substituted acids.

SYNTHESIS OF URETHANE N-CARBOXYANHYDRIDES OF β-AMINO ACIDS

Jacques Huck, Mary Mc Kiernan, Jean-Alain Fehrentz, Marie-Louise Roumestant, Jean Martinez
Laboratoire des Aminoacides, Peptides et Protéines, UMR 5810 CNRS-UM1-UM2, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.

N-Urethane *N*-carboxyanhydrides from α-amino acids (UNCAs) are attractive target molecules for medicinal chemists as they have incorporate into their structure an activated carboxylate group and a protected amino group. Such molecules are highly reactive and are useful for stepwise peptide synthesis, solid phase synthesis and synthesis of a large variety of amino acid derivatives (S. Bouifraden et al., Amino Acids, 1999).
Recently considerable attention has been directed towards the understanding of biological activity and structural implication of β-amino acids and their derivatives. β-Amino acids are found as components of peptides and natural products with antibiotic, antifungal, cytotoxic properties. Therefore the intent of this investigation was to synthesise *N*-urethane *N*-carboxyanhydride of b-amino acids and to study their reactivity.

* R1= R2 = H
* R1 = Me ; R2 = H
* R1 = H ; R2 = Me