DOI: 10.1002/adsc.201200423

# Racemization-Free Chemoenzymatic Peptide Synthesis Enabled by the Ruthenium-Catalyzed Synthesis of Peptide Enol Esters *via* Alkyne-Addition and Subsequent Conversion Using Alcalase-Cross-Linked Enzyme Aggregates

Hilmar Schröder,[a,b] Gernot A. Strohmeier,[b] Mario Leypold,[a] Timo Nuijens,[c] Peter J. L. M. Quaedflieg,[c] and Rolf Breinbauer[a,b,*]

[a] Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 9, 8010 Graz, Austria
Fax: (+43)-316-873-32402
(homepage: www.orgc.tugraz.at); e-mail: breinbauer@tugraz.at
[b] ACIB – Austrian Centre of Industrial Biotechnology GmbH, Petersgasse 14, 8010 Graz, Austria
[c] DSM Innovative Synthesis, P. O. Box 18, 6160 MD Geleen, The Netherlands

Received: May 14, 2012; Revised: December 7, 2012; Published online: February 28, 2013

Dedicated to Professor Herfried Griengl on the occasion of his 75th birthday.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201200423.

**Abstract:** The *C*-terminal activation of peptides as prerequisite for the formation or ligation of peptide fragments is often associated with the problem of epimerization. We report that ruthenium-catalyzed alkyne addition with (+)-2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane as ligand allows the racemization-free synthesis of peptide enol esters tolerating a wide range of functional groups. The transformation can be performed in a variety of different solvents addressing the solubility issues imposed by peptides with varying amino acid side chain patterns. We show that peptide enol esters with an amide motif in the enol moiety are excellent acyl donors for the peptide condensation with other peptide fragments in organic solvents using serine endopeptidase subtilisin A as catalyst. The reported combination of transition metal catalysis with enzymatic peptide ligations adds an important tool for the racemization-free synthesis and ligation of peptides which is compatible even with unprotected amino acid side chains.

**Keywords:** alcalase; biocatalysis; enol esters; peptide synthesis; ruthenium

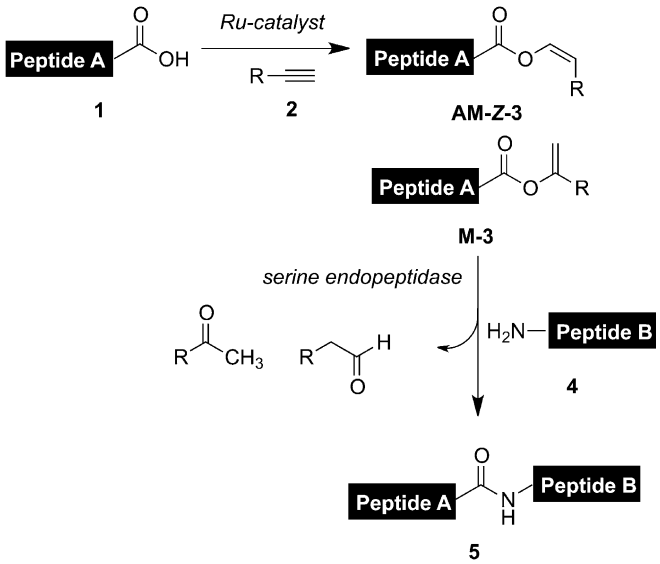
## Introduction

In recent years, the scalable synthesis of peptides, ranging from simple dipeptides to oligopeptides up to 50 amino acids in length, has gained significant importance, as such products have been increasingly investigated and marketed in the pharmaceutical, cosmetics and nutrition industry.[1] Although several excellent methods and reagents for the assembly of peptides are available, the issue of racemization-free fragment coupling remains a problem without a general solution.[2] One attractive method is the chemoenzymatic synthesis of peptides using proteases,[3] where either suitable reaction conditions shift the equilibrium to the coupling product,[4] or activated esters[5] or thioesters[6] are used. In principle, chemoenzymatic peptide synthesis is distinguished by the advantage of enzymatic chemoselectivity, which allows the conversion of non-side chain protected peptides and the absolute absence of racemization. We have recently reported in a series of publications that the serine endopeptidase subtilisin A (commercially available as Alcalase) is a practical enzyme for the preparation of oligopeptides.[7] One challenge we identified is the racemization-free preparation of activated peptide esters which are suitable substrates for this approach. In this manuscript we report about a convenient and atom-economical method to prepare peptide enol esters without racemization using transition metal catalysis and their subsequent use for enzymatic peptide synthesis. This approach takes advantage of the so far rather unexplored opportunities offered by the combination of homogeneous transition metal catalysis and enzymatic catalysis.[8]

## Results and Discussion

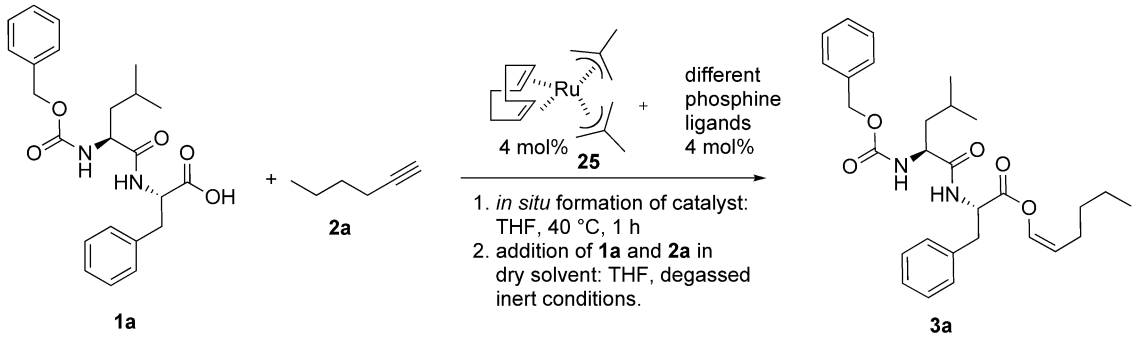
Activation of a peptide's *C*-terminal carboxylic acids for chemoenzymatic condensation with peptide nucleophiles has so far relied on the chemical or enzymatic synthesis of thioesters, or the chemical synthesis of activated esters. Most of these strategies involve the activation of the *C*-terminal carbonyl group followed by addition of a nucleophile – a process which is often accompanied by a certain amount of racemization. We[7] and others[9] could demonstrate that the activation of peptides as Cam esters (Cam = carboxamidomethyl), in which the carboxyl group serves as a nucleophile in the reaction with the strong electrophile iodoacetamide, is a viable alternative method for *C*-terminal activation. However, this method is less suitable for unprotected peptides featuring nucleophilic amino acid side chains. In order to provide a more general solution to this problem, we envisaged that the addition of the *C*-terminal carboxyl group across a terminal alkyne moiety would result in the atom-economical production of enol esters, which have been recognized as ideal reagents in enzymatic acylations, although so far only for simple and more or less unfunctionalized acids.[10,11] These peptide enol esters would then act as substrates for peptide coupling with a serine endopeptidase (Scheme 1).




**Scheme 1.** General scheme for the preparation of peptide enol esters and their use in enzymatic coupling with peptide nucleophiles.

The key challenge in our approach outlined in Scheme 1 was therefore to develop a mild, racemization-free method for the synthesis of enol esters of quite complex peptide fragments which are significantly more prone to racemization than single amino acids.[2d] Among several methods considered, we identified the reports by Watanabe,[12] Dixneuf,[13] and Gooßen[14] for the Ru-catalyzed addition of carboxylic acids to alkynes as rewarding starting points for our investigations.[15,16] Indeed, Dixneuf and Gooßen disclosed the synthesis of amino acid enol esters without racemization.[13,14,17] We could positively reproduce this finding for single amino acid substrates, but we observed substantial epimerization of the *C*-terminal amino acid residue when dipeptide Z-Leu-Phe-OH (**1a**) was subjected to the reported conditions (Z = benzyloxycarbonyl). In our original plan, we would have preferred to produce the Markovnikov-enol ester **M-3** (Scheme 1), which is accessible *via* Gooßen's method, as a substrate for the enzymatic reaction, as upon conversion a less electrophilic ketone side product would form. However, our efforts in optimizing the reaction conditions did not result in a significant reduction of epimerization, probably because the addition of a base appears to be an important component to achieve Markovnikov-selectivity.[14] Gratifyingly, our parallel efforts in optimization studies for the production of the anti-Markovnikov product **AM-*Z*-3** were much more rewarding.

We chose Z-Leu-Phe-OH (**1a**) as a suitable test substrate, as Phe is known as the amino acid to be most prone towards racemization,[18] so any successful reagent combination for this substrate should be suitable as well for peptides bearing less challenging *C*-terminal amino acid residues (Scheme 2, Figure 1, Table 1). In an extensive screening of different types




**Scheme 2.** Ru-catalyzed addition of alkynes **2** to *N*-protected amino acids **1**.

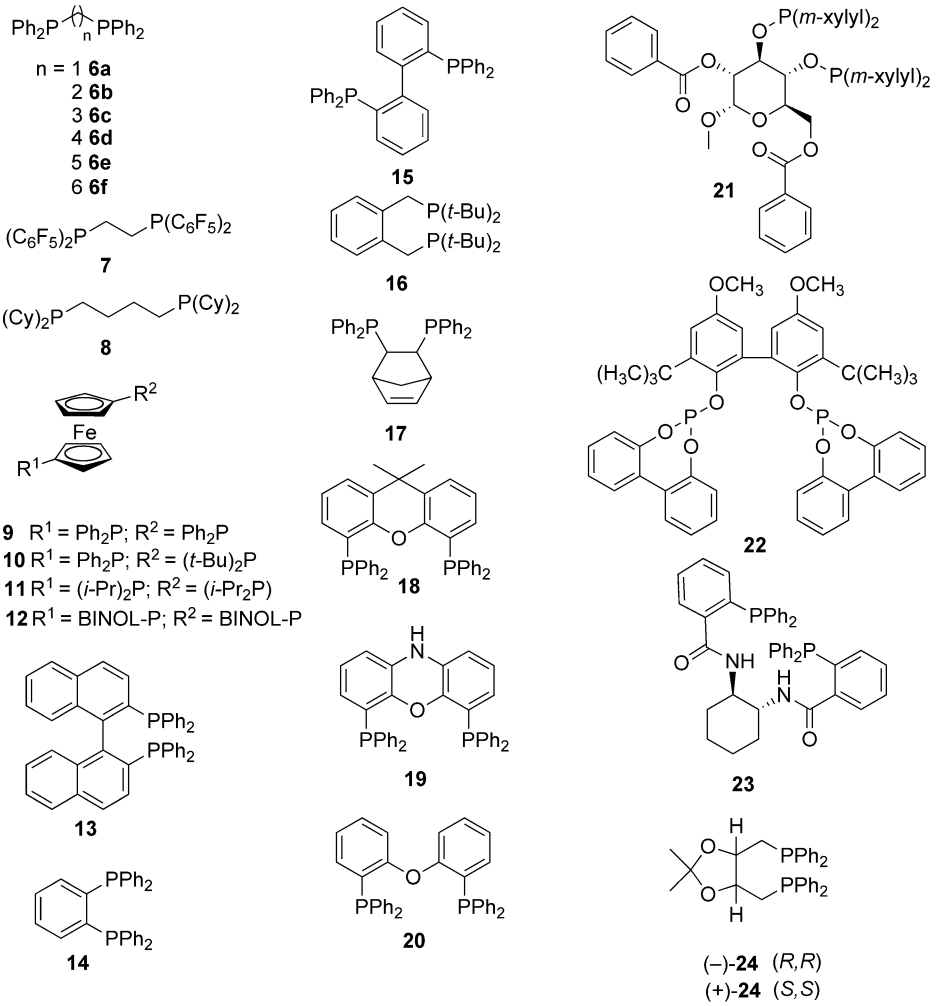



**Figure 1.** Structures of bidentate phosphorus ligands **6**–**24** used for the Ru-catalyzed alkyne addition to Z-Leu-Phe-OH (**1a**).

of ligands in which we measured the conversion after 24 h using *in situ* prepared Ru complexes,[19] we recognized that the bite angle of the bidentate phosphine ligand imposes an important influence on the reaction, as has been suggested before.[13c] In the homologous series of bis(diphenylphosphino)alkane ligands **6a–f**, dppb ligand **6d** showed the highest activity leading to complete conversion of substrate in 24 h, and with a high selectivity towards the *Z*-anti-Markovnikov-product **AM-Z-3a**. Testing ligands **7**–**23** which possess a more rigid backbone, gave mixed results, but no improvement in comparison with dppb **6d**.

However, 2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane (DIOP) ligand **24**, which can be regarded as a conformationally restricted version of dppb **6d** gave superior results. Interestingly, (+)-**24** showed higher activity than (−)-**24** indicating a possible cooperative interaction between the chiral catalyst and the chiral substrate [89% conversion after 3 h in THF with (+)-**24** *vs.* 40% conversion after 3 h in THF with (−)-**24**]. Our studies also allowed us to conclude that a strong electronic effect of the bidentate phosphine ligands is exerted, leading to an improvement of conversion by shifting towards more electron-poor phosphines (see entries 2 and 7 or, respectively, 4 and 8, Table 1).

As the solubility of peptide substrates varies with the amino acid side chains, the question which solvents were most suitable for the alkyne addition required special attention. Ideally, a set of several solvent systems should be available addressing the challenges associated with varying solubilities imposed by different peptide sequences. For the enol ester formation with isolated catalysts **26** and **27**[20] derived from our best ligands dppb **6d** and (+)-DIOP [(+)-**24**], we observed strong solvent effects on both the reaction rate as well as on the degree of racemization (Scheme 3, Table 2).[21] In general, slightly polar solvents, such as THF, DME, chloroform, or 1,4-dioxane seemed to provide the best results. Surprisingly, using polar protic solvents such as MeOH, EtOH and 2-propanol led to the fastest conversions and the lowest

**Table 1.** Screening of different types of bidentate phosphorus ligands **6–24** in the Ru-catalyzed addition of 1-hexyne (**2a**) to Z-Leu-Phe-OH (**1a**) in THF at 40 °C (conc. 0.5 mmol $L^{-1}$).

| Entry | Ligand[a] | Conversion [%][b] | **AM-*Z*-3a/AM-*E*-3a/M-3a**[c] |
|---|---|---|---|
| 1 | **6a** | 43 | 93/–/6 |
| 2 | **6b** | 18 | 41/–/59 |
| 3 | **6c** | 57 | 29/–/71 |
| 4 | **6d** | 97 | 98/–/2 |
| 5 | **6e** | 57 | 84/–/16 |
| 6 | **6f** | 43 | 53/–/47 |
| 7 | **7** | 51 | 91/–/9 |
| 8 | **8** | 77 | 25/–/75 |
| 9 | **9** | 54 | 93/–/7 |
| 10 | **10** | 22 | 19/–/81 |
| 11 | **11** | 40 | 78/–/22 |
| 12 | **12** | 5 | nd |
| 13 | **13** | 46 | 97/–/3 |
| 14 | **14** | 15 | 83/–/17 |
| 15 | **15** | 78 | 99/–/1 |
| 16 | **16** | 18 | 64/–/36 |
| 17 | **17** | 57 | 98/–/2 |
| 18 | **18** | 10 | nd |
| 19 | **19** | 5 | nd |
| 20 | **20** | 56 | 99/–/1 |
| 21 | **21** | 17 | 78/–/22 |
| 22 | **22** | 21 | 75/–/25 |
| 23 | **23** | 8 | nd |
| 24 | (−)-**24** | 98 | 96/–/4 |
| 25 | (+)-**24** | 99 | > 99/–/ < 1 |

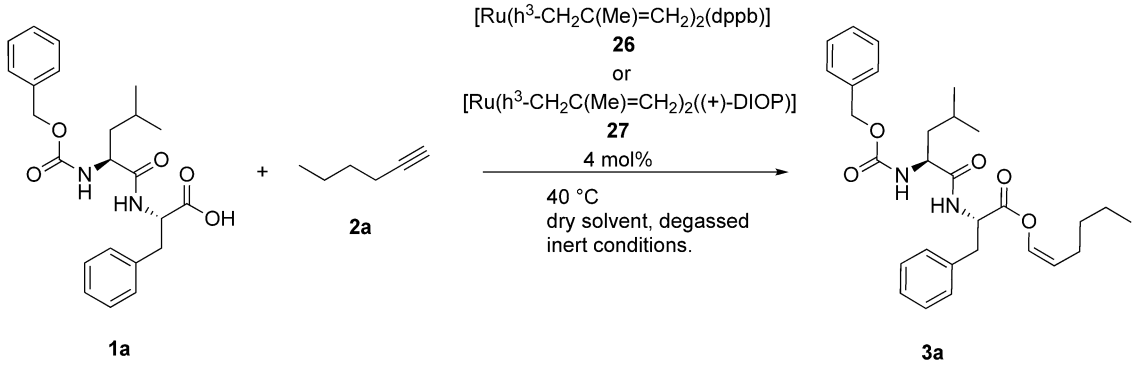
[a] 4 mol% of ligand used based on **1a**.
[b] Determined after 24 h.
[c] Determined by reversed-phase HPLC. nd = not determined.

amounts of racemization. Interestingly, we did not observe any side products resulting from transesterification of **3a** in the presence of these alcohols. With (+)-**24** as ligand, we could produce **AM-Z-3a** in 85–90% isolated yield and with less than 0.2% racemization at the *C*-terminal Phe residue, when using MeOH, EtOH, 1-propanol, 2-propanol or chloroform as solvents (entries 17–20, 22, Table 2).

Notably, a solvent which leads to a high degree of racemization, such as dichloromethane, can be integrated in a suitable solvent system if 2-propanol is added as an additive (entries 10 and 24, Table 2). These results make us optimistic that it will be possible to identify an appropriate solvent combination for almost any substrate.

With (+)-DIOP **24** as the best ligand and 2-propanol as the best solvent, we had conditions at hand with which we set out to explore the scope of this reaction for different peptide substrates **1a–j** and alkyne partners **2a–d** (Scheme 4, Table 3). We were pleased that our method allowed the quantitative and racemization-free enol esterification of a great variety of peptide substrates with several functionalized alkynes. Notably, the method tolerates unprotected Ser and Tyr in substrates (entries 11 and 12). Also the S-containing amino acid Met was tolerated, although for complete conversion we had to add a second amount of catalyst (entry 15). Fortunately, no or negligible racemization was encountered in the Ru-catalyzed alkyne addition using (+)-DIOP **24** as ligand.

Having now established a general and efficient access to peptide enol esters **3a–o**, we were keen to investigate their suitability in enzymatic condensation reactions. As a model reaction, we studied the coupling of Z-Leu-Phe-enol ester **3a** with H-Phe-$NH_2$ (**4a**) using Alcalase cross-linked enzyme aggregates (Alcalase-CLEA) as catalyst in THF. Unfortunately, but not unexpectedly, we observed a considerable amount of an imine side product, which resulted from the reaction of H-Phe-$NH_2$ (**4a**) with the aldehyde released upon reaction of our substrate **3a** with the serine endopeptidase in the active center. In principle, one could increase the coupling yield by increasing the amount of amine reagent **4a**, but this would result in a waste of valuable starting material and an increase of the purification effort during work-up. We




**Scheme 3.** Ru-catalyzed addition of 1-hexyne (**2a**) to Z-Leu-Phe-OH (**1a**).

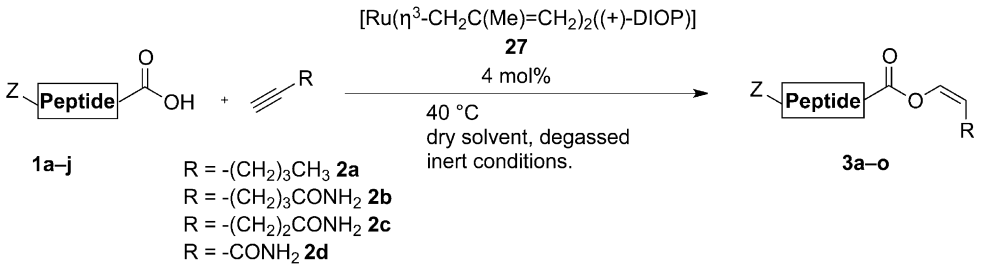
  

**Table 2.** Screening of various solvents in the Ru-catalyzed addition of 1-hexyne (**2a**) to Z-Leu-Phe-OH (**1a**, 0.5 M) at 40 °C.

| Entry | Catalyst | Solvent | Conversion [%] after 1 h | 3 h | 24 h[a] | Yield [%] | D-Phe [%][b] |
|---|---|---|---|---|---|---|---|
| 1 | **26** | THF | nd | 36 | 95 | 68 | 0.6 |
| 2 | **26** | 2-Me-THF | nd | 70 | 98[c] | nd | 0.3 |
| 3 | **26** | 1,4-dioxane | nd | 55 | 97 | nd | 0.4 |
| 4 | **26** | DME | nd | 55 | 92 | nd | 5.1[d] |
| 5 | **26** | DMF | nd | 13 | 36 | nd | nd |
| 6 | **26** | NMP | nd | 15 | 29 | nd | nd |
| 7 | **26** | DMSO | nd | <1 | 1 | nd | nd |
| 8 | **26** | sulfolane | nd | 65 | 86 | nd | 3.5[d] |
| 9 | **26** | MeCN | nd | <1 | 1 | nd | nd |
| 10 | **26** | $CH_2Cl_2$ | nd | 54 | 90 | nd | 13.2[d] |
| 11 | **26** | chloroform | nd | 64 | 99 | 85 | 2.0 |
| 12 | **26** | acetone | nd | 52 | 82 | nd | 14.2[d] |
| 13 | **26** | methanol[f] | nd | 25 | 65 | nd | 0.5 |
| 14 | **26** | ethanol | nd | 85 | 98[c] | nd | 0.7 |
| 15 | **26** | 2-propanol | nd | 98 | nd | 84 | 0.3 |
| 16 | **26** | *tert*-butyl alcohol[f] | nd | 65 | 92 | nd | 1.9 |
| 17 | **27** | methanol[f] | 26 | 51 | 95 | 85 | <0.1 |
| 18 | **27** | ethanol | 73 | 91 | nd | 86 | <0.1[e] |
| 19 | **27** | 1-propanol | 70 | 91 | nd | 87 | <0.1[e] |
| 20 | **27** | 2-propanol | 99 | nd | nd | 88 | <0.1[g] |
| 21 | **27** | MTBE/DMF (8:2) | 9 | 42 | 99 | 90 | 0.3 |
| 22 | **27** | chloroform | 66 | 98 | nd | 87 | 0.2[e] |
| 23 | **27** | $CH_2Cl_2$ | 39 | 73 | 99 [h] | 88 | 1.5[h] |
| 24 | **27** | $CH_2Cl_2$/2-propanol (9:1) | 72 | 98 | nd | 89 | 0.7[i] |

[a] Sum of isomers.
[b] After 24 h reaction time and upon subsequent hydrolysis with 6 N HCl.
[c] After 18 h.
[d] Determined after 48 h.
[e] Determined after 6 h.
[f] Reaction carried out at a concentration of 0.25 mmol·L$^{-1}$.
[g] Determined after 1 h.
[h] Determined after 8 h.
[i] Determined after 3 h.

reasoned that we could avoid imine formation by addressing two issues. First of all, we aimed at improving the rate of enzymatic peptide coupling, as this reaction is kinetically independent from the slower and unwanted imine formation. Taking into account that Alcalase is an endoprotease recognizing amino acids at both directions in its active site, we speculated that the introduction of amide motifs within the enol ester moiety would allow a better recognition of our substrates by the enzyme and ultimately lead to faster reactions. To verify this hypothesis, we tested Z-Leu-Phe enol esters **3b** and **3c** containing amide bonds and compared them with substrate **3a** and Z-Leu-Phe-Cam ester **3p** (Scheme 5). We were gratified to see that indeed the enol esters **3b** and **3c** containing an amide motif in the enol moiety performed much better than our standard hexenyl ester **3a**, with **3b** being even slightly better than the corresponding Cam ester **3p** (Table 4, Figure 2). The formation of tetrapeptide Z-Leu-Phe-Phe-Phe-$NH_2$ can be ex-



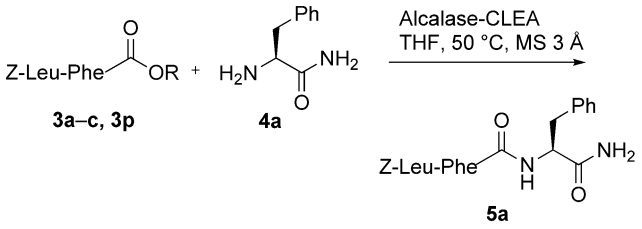

**Scheme 4.** Z-anti-Markovnikov Ru-catalyzed addition of alkynes **2** to peptide fragments **1**.

**Table 3.** Racemization-free Ru-catalyzed synthesis of amino acid and peptide enol esters **3** using [Ru($\eta^3$-$CH_2C(Me)=CH_2$)$_2$((+)-DIOP)] (**27**) at 40 °C in 2-propanol.

| Entry | Peptide | Alkyne | Time [h] | Conversion [%] | Yield [%] | D-AA [%][a] |
|---|---|---|---|---|---|---|
| 1 | Z-Leu-Phe-OH **1a** | **2a** | 1 | 99 | 88 **3a** | <0.1 |
| 2 | Z-Leu-Phe-OH **1a** | **2b** | 2 | 98[b] | 80 **3b** | <0.1 |
| 3 | Z-Leu-Phe-OH **1a** | **2c** | 2 | 97[b] | 82 **3c** | <0.1 |
| 4 | Z-Leu-Phe-OH **1a** | **2d** | 24 | 0 | nd | nd |
| 5 | Z-Leu-Ala-OH **1b** | **2a** | 1 | 99 | 92 **3d** | 0.1 |
| 6 | Z-Leu-Ala-OH **1b** | **2b** | 1 | 99[b] | 89 **3e** | 0.2 |
| 7 | Z-Phe-Leu-OH **1c** | **2a** | 1 | 99 | 93 **3f** | 0.3 |
| 8 | Z-Phe-Leu-OH **1c** | **2b** | 1 | 98[b] | 92 **3g** | 0.1 |
| 9 | Z-Phe-Leu-Ala-OH **1d** | **2a** | 1 | 99 | 87 **3h** | 0.1 |
| 10 | Z-Phe-Leu-Ala-OH **1d** | **2b** | 1 | 97[b] | 86 **3i** | 0.1 |
| 11 | Boc-Phe-Tyr-OH **1e** | **2a** | 3 | 99 | 86 **3j** | 0.2 |
| 12 | Z-Ile-Ser-OH **1f** | **2a** | 24 | 97[c] | 83 **3k** | 0.1 |
| 13 | Z-Phe-Pro-OH **1g** | **2b** | 5 | 97[b] | 78 **3l** | 0.3 |
| 14 | Z-Phe-Val-OH **1h** | **2b** | 2 | 98[b] | 83 **3m** | 0.2 |
| 15 | Z-Phe-Met-OH **1i** | **2a** | 7 | 97[d] | 87 **3n** | 0.7 |
| 16 | Z-Phe-OH **1j** | **2a** | 24 | 99[e] | 89 **3o** | <0.1 |

[a] Amount of *C*-terminal racemization determined after the indicated reaction time upon hydrolysis with 6N HCl.
[b] Bis-addition products up to 2% relative to product and starting material were detected.[22]
[c] Ethanol used as solvent, 1.5% ethyl ester detected.
[d] Addition of 4 mol% of catalyst after 3 h.
[e] Catalyst **26** used.




**Scheme 5.** Alcalase-catalyzed condensation of peptide enol esters **3** with H-Phe-$NH_2$ (**4a**).

plained by the high transamidation activity of Alcalase in case of *N*-terminal phenylalanine amide residues to catalyze a chain elongation of previously formed **4a**. In all other cases, this secondary reaction was slower or even not observed.

Based on the promising results of the kinetic study with type-**3b** peptide enol esters, we went on to perform coupling reactions with larger amino fragments.[7f] Fortunately, peptide enol esters **3b** and **3e** were efficiently converted to the corresponding tetra- or 7-mer in the presence of Alcalase and the appropriate amino fragment (Scheme 6, Table 5). The results indicate that the coupling protocol is fully applicable with longer peptide fragments.

As a second line of thought in the effort to avoid imine formation, we speculated that the molecular sieves added to remove water might also serve as a catalyst for the undesired imine formation, as its acidic properties are well recognized.[23] Our efforts to replace the molecular sieves in the reaction mixture by other dehydrating agents such as alumina, $MgSO_4$, or $Na_2SO_4$ were not rewarded, as these reagents either even aggravated the imine formation or turned out to be inefficient in removing residual water. Fortunately, when using a Soxhlet-type set up (Figure 1 in the Supporting Information), in which the molecu-

**Table 4.** Comparison of various esters **3** in the Alcalase-catalyzed peptide condensation with H-Phe-$NH_2$ (**4a**) in THF at 50 °C.

| Entry | Peptide ester | Conversion[%][a] | Product composition[b] |
|---|---|---|---|
| 1 | Z-Leu-Phe-OCam **3p** | 98 | 87:11 (–) |
| 2 | Z-Leu-Phe-O(*Z*)-hex-1-enyl **3a** | 70 | 63:7 (2) |
| 3 | Z-Leu-Phe-O(*Z*)-6-amino-6-oxohex-1-enyl **3b** | 99 | 91:8 (3) |
| 4 | Z-Leu-Phe-O(*Z*)-5-amino-5-oxopent-1-enyl **3c** | 93 | 85:8 (<1) |

[a] Determined after 3 h reaction time by reversed-phase HPLC.
[b] Product composition Z-Leu-Phe-Phe-$NH_2$ (**5a**):Z-Leu-Phe-Phe-Phe-$NH_2$ (imine formation relative to starting material + products).

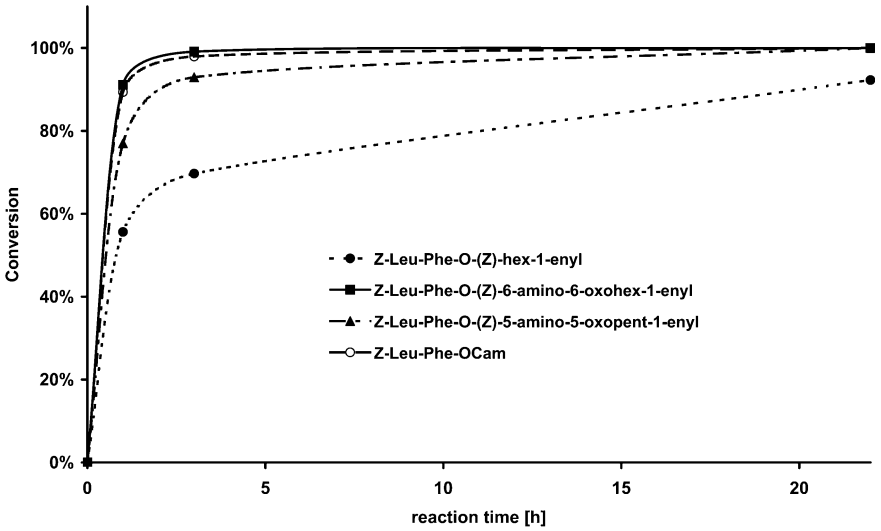
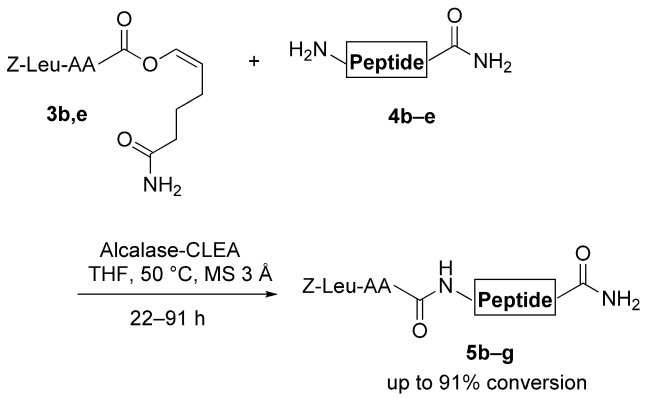



**Figure 2.** Kinetic comparison of Alcalase-catalyzed peptide coupling of various activated esters **3** with H-Phe-$NH_2$ (**4a**) in THF at 50 °C.




**Scheme 6.** Alcalase-catalyzed condensation of peptide enol esters **3b** and **3e** with amino fragments (**4b–e**).

lar sieves did not get into contact with the amino nucleophile and aldehyde side product anymore, while removing water from the THF azeotrope, imine formation was reduced to a negligible amount, confirming our hypothesis about the catalytic effect of the molecular sieves in imine formation (Scheme 7). Combining these two insights allowed us to define a reaction protocol which leads to the high-yielding chemoenzymatic synthesis of oligopeptides. For our test reaction, we obtained Z-Leu-Phe-Phe-$NH_2$ (**5a**) in 91% isolated yield, demonstrating that our now racemization-free protocol toward peptide enol esters gives us access to peptide acyl donors which can be efficiently condensed in our Alcalase peptide coupling platform.[7]

## Conclusions

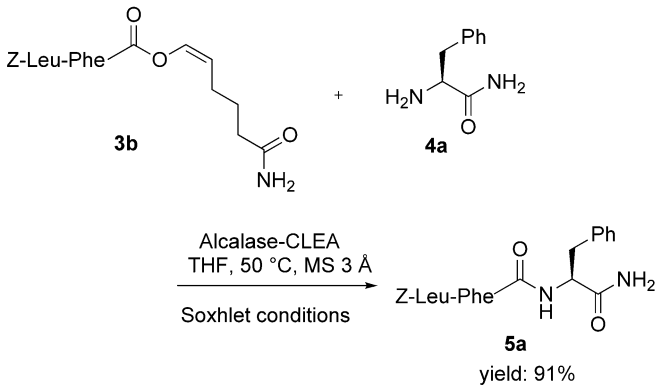
In conclusion, we have demonstrated that under appropriate conditions the Ru-catalyzed addition of alkynes using (+)-DIOP as a ligand is a convenient method for the racemization-free preparation of *C*-terminal peptide enol esters, which tolerates a considerable number of unprotected amino acids. These peptide enol esters are excellent substrates in chemoenzymatic peptide coupling reactions using Alcalase.

**Table 5.** Alcalase-catalyzed peptide ligation using enol esters **3** and various amino fragments **4** at 50 °C in THF.

| Entry | Enol ester | Amino fragment | Time [h] | Conversion [%] | Isolated yield [%][b] | Purity [%][c] |
|---|---|---|---|---|---|---|
| 1 | **3b** | H-Ala-Val-$NH_2$[a] **4b** | 91 | 61 | 62 **5b** | 94 |
| 2 | **3b** | H-Leu-Phe-$NH_2$ **4c** | 22 | 71 | 30 **5c** | 87 |
| 3 | **3e** | H-Ala-Val-$NH_2$[a] **4b** | 22 | 84 | 78 **5d** | 82 |
| 4 | **3e** | H-Gly-Phe-$NH_2$ **4d** | 22 | 74 | 41 **5e** | 80 |
| 5 | **3e** | H-Leu-Phe-$NH_2$ **4c** | 22 | 91 | 51 **5f** | 98 |
| 6 | **3e** | H-Phe-Val-Leu-Met-Gly-$NH_2$[a] **4e** | 91 | 63 | 27 **5g** | 92 |

[a] Used as hydrochlorides.
[b] Purified by preparative reversed-phase HPLC.
[c] Determined by reversed-phase HPLC at 210 nm.




**Scheme 7.** Alcalase-catalyzed coupling of **3b** and H-Phe-$NH_2$ (**4a**) under Soxhlet conditions.

Amide recognition motifs within the enol ester moiety are recognized by the enzyme and enable more favorable reaction rates and coupling yields. With this universal method for making enol esters available, by taking advantage of the power of Ru-catalyzed alkyne addition, we have expanded the scope of enzyme-catalyzed peptide fragment condensation. We are confident that such a strategy in which the advantages of homogeneous catalysis and biocatalysis are combined, will offer many more rewarding opportunities. Activities towards this goal are currently being pursued in our laboratories.

## Experimental Section

### General Procedure for Ru-Catalyzed Enol Ester Formation of Peptides using 1-Hexyne (2a)

All experiments were carried out under argon in an oven-dried Schlenk tube containing a magnetic stirring bar. The vessel was charged with Z-Leu-Phe-OH (**1a**) (103.1 mg, 0.25 mmol, 1.0 equiv), Ru catalyst **27** (7.1 mg, 0.01 mmol, 0.04 equiv.) and dry 2-propanol (0.5 mL) degassed prior to use. The materials were dissolved by a few seconds heating to reflux temperature and then the vessel was immediately cooled and immersed in an oil bath preheated to 40 °C. Then degassed 1-hexyne (**2a**) (34 μL, 0.3 mmol, 1.2 equiv.) was added with a Hamilton syringe to the reaction solution and the mixture was stirred at 40 °C. After the appropriate conversion had been reached the solution was concentrated under vacuum. The solid residue was then redissolved in dichloromethane, and the solution was concentrated in the presence of the same quantity of silica gel and the resulting residue was subjected to chromatographic purification. The fractions containing the desired product were pooled and concentrated under vacuum. The residue was redissolved in dichloromethane and subsequently evaporated (3×) and the product **3a** was finally dried at 0.02 mbar; yield: 102 mg (82%); $R_f$ 0.30 (cyclohexane/ethyl acetate 5:1). $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 0.90$–$0.92$ [m, 9H, -$CH_2$-C$H_3$, -CH-($CH_3$)$_2$], 1.26–1.72 [m, 7H, -NH-CH-C$H_2$-CH-($CH_3$)$_2$, -$CH_2$-C$H$-($CH_3$)$_2$, -$CO_2$-CH=CH-$CH_2$-($CH_2$)$_2$-$CH_3$], 2.04–2.11 (m, 2H, -CH=CH-C$H_2$-$CH_2$-), 3.15–3.17 (m, 2H, $C_6H_5$-C$H_2$-CH-NH), 4.17–4.22 [m, 1H, NH-C$H$($CH_2$-CH($CH_3$)$_2$)-CO-NH], 4.92–4.99 [m, 2H, CO-NH-C$H$($CH_2$-Ph)-$CO_2$-, -$CO_2$-CH=C$H$-$CH_2$-], 5.06–5.15 (m, 3H, HN-$CO_2$-C$H_2$-$C_6H_5$, -N$H$-), 6.51 (d, 1H, $^3J_{H,H} = 7.2$ Hz, -N$H$-), 6.97 (d, 1H, $^3J_{H,H} = 6.3$ Hz, $CO_2$-C$H$=CH-$CH_2$), 7.09–7.36 (m, 10H, $H_{Ar}$); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 14.0$ [$CH_3$-($CH_2$)$_3$-CH=CH-$O_2$C], 22.1, 22.3, 23.0, 24.2, 24.8, 31.3 ($C_{aliphatic}$), 38.1 ($C_6H_5$-$CH_2$-CH-NH-), 41.4 [NH-CH-$CH_2$-CH-($CH_3$)$_2$], 53.2, 53.6 (2×NH-$CH$), 67.3 (HN-$CO_2$-$CH_2$-$C_6H_5$), 115.8 ($CO_2$-CH=$CH$-$CH_2$-), 127.4 ($C_{Ar}$), 128.2 (2×$C_{Ar}$), 128.4 ($C_{Ar}$), 128.7 (2×$C_{Ar}$), 128.8 (2×$C_{Ar}$), 129.4 (2×$C_{Ar}$), 133.6 ($CO_2$-$CH$=CH-$CH_2$-), 135.5 ($C_{q,Ar}$), 136.3 ($C_{q,Ar}$), 156.2 (NH-$CO_2$-$CH_2$-$C_6H_5$), 168.7 ($C_q$), 171.9 ($C_q$); HR-MS: $m/z = 517.2716$, calcd. for $C_{29}H_{38}N_2O_5$ [MNa]$^+$: 517.2678; $[\alpha]_D^{25}$: −13.1° (*c* 0.5, chloroform).

## Acknowledgements

*This work has been supported by the Federal Ministry of Economy, Family and Youth (BMWFJ), the Federal Ministry of Traffic, Innovation and Technology (BMVIT), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol and ZIT – Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG. We thank Bernhard Wölfl for skilful assistance in experimental work.*

## References

[1] a) P. Vlieghe, V. Lisowski, J. Martinez, M. Khrestchatisky, *Drug Discovery Today* **2010**, *15*, 40–56; b) A. K. Sato, M. Viswanathan, R. B. Kent, C. R. Wood, *Curr. Opin. Biotechnol.* **2006**, *17*, 638–642; c) T. Bruckendorfer, O. Marder, F. Albericio, *Curr. Pharm. Biotechnol.* **2004**, *5*, 29–43; d) B. L. Bray, *Nat. Rev. Drug Discovery* **2003**, *2*, 587–593.

[2] For authoritative reviews, see a) E. Valeur, M. Bradley, *Chem. Soc. Rev.* **2009**, *38*, 606–631; b) C. A. G. N. Montalbetti, V. Falque, *Tetrahedron* **2005**, *61*, 10827–10852; c) S.-Y. Han, Y.-A. Kim, *Tetrahedron* **2004**, *60*, 2447–2467; d) *Synthesis of Peptides and Peptidomimetics*, Vol. E22, (Eds. M. Goodman, A. Felix, L. Moroder, C. Toniolo), Thieme, Stuttgart, **2002**; e) J. M. Humphrey, A. R. Chamberlin, *Chem. Rev.* **1997**, *97*, 2243–2246. Some recent examples of innovative coupling strategies: f) V. Declerck, P. Nun, J. Martinez, F. Lamaty, *Angew. Chem.* **2009**, *121*, 9482–9485; *Angew. Chem. Int. Ed.* **2009**, *48*, 9318–9321.

[3] Reviews: a) S. Tsukiji, T. Nagamune, *ChemBioChem* **2009**, *10*, 787–798; b) M. Yagasaki, S.-i. Hashimoto, *Appl. Microbiol. Biotechnol.* **2008**, *81*, 13–22; c) D. Kumar, T. C. Bhalla, *Appl. Microbiol. Biotechnol.* **2005**, *68*, 726–736; d) D.-F. Tai, *Curr. Org. Chem.* **2003**, *7*, 515–554; e) F. Bordusa, *Chem. Rev.* **2002**, *102*, 4817–4867.

[4] a) A. L. Margolin, A. M. Klibanov, *J. Am. Chem. Soc.* **1987**, *109*, 3802–3804; b) N. Wehofsky, C. Wespe, V.

Čerofsky, A. Pech, E. Hoess, R. Rudolph, F. Bordusa, *ChemBioChem* **2008**, *9*, 1493–1499; c) R. Haddoub, M. Dauner, F. A. Stefanowicz, V. Barattini, N. Laurent, S. L. Flitsch, *Org. Biomol. Chem.* **2009**, *7*, 665–670; d) R. V. Ulijn, N. Bisek, P. J. Halling, S. L. Flitsch, *Org. Biomol. Chem.* **2003**, *1*, 1277–1281; e) R. V. Ulijn, B. Baragana, P. J. Halling, S. L. Flitsch, *J. Am. Chem. Soc.* **2002**, *124*, 10988–10999.

[5] Examples: a) F. Weygand, W. Steglich, *Angew. Chem.* **1961**, *73*, 757; b) X.-G. Li, L. T. Kanerva, *Org. Lett.* **2006**, *8*, 5593–5596.

[6] N. Wehofsky, N. Koglin, S. Thust, F. Bordusa, *J. Am. Chem. Soc.* **2003**, *125*, 6126–6133.

[7] a) T. Nuijens, C. Cusan, A. C. M. Schepers, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *J. Mol. Catal. B: Enzym.* **2011**, *71*, 79–84; b) T. Nuijens, E. Piva, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *Adv. Synth. Catal.* **2011**, *353*, 1039–1044; c) T. Nuijens, C. Cusan, T. J. G. M. van Dooren, H. M Moody, R. Merkx, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *Adv. Synth. Catal.* **2010**, *352*, 2399–2404; d) T. Nuijens, C. Cusan, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *J. Org. Chem.* **2009**, *74*, 5145–5150; e) T. Nuijens, J. A. W. Kruijtzer, C. Cusan, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *Tetrahedron Lett.* **2009**, *50*, 2719–2721; for application in coupling larger peptide fragments, see: f) T. Nuijens, A. H. M. Schepers, C. Cusan, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp, P. J. L. M. Quaedflieg, *Adv. Synth. Catal.* **2013**, *355*, 287–293.

[8] Selected examples: a) E. Burda, W. Hummel, H. Gröger, *Angew. Chem.* **2008**, *120*, 9693–9696; *Angew. Chem. Int. Ed.* **2008**, *47*, 9551–9554; b) F. G. Mutti, A. Orthaber, J. H. Schrittwieser, J. G. de Vries, R. Pietschnig, W. Kroutil, *Chem. Commun.* **2010**, *46*, 8046–8048; c) V. Gauchot, W. Kroutil, A. R. Schmitzer, *Chem. Eur. J.* **2010**, *16*, 6748–6751; d) A. Boffi, S. Cacchi, P. Ceci, R. Cirilli, G. Fabrizi, A. Prastaro, S. Niembro, A. Shafir, A. Vallribera, *ChemCatChem* **2011**, *3*, 247–253.

[9] For example: a) P. Kuhl, U. Zacharias, H. Burckhardt, H.-D. Jakubke, *Monatsh. Chem.* **1986**, *117*, 1195–1204; b) S. M. A. Salam, K.-i. Kagawa, K. Kawashiro, *Tetrahedron: Asymmetry* **2006**, *17*, 22–29.

[10] Selected examples: a) Y.-F. Wang, C-H. Wong, *J. Org. Chem.* **1988**, *53*, 3127–3129; b) Y. Kita, Y. Takebe, K. Murata, T. Naka, S. Akai, *J. Org. Chem.* **2000**, *65*, 83–88; c) H. K. Weber, J. Zuegg, K. Faber, J. Pleiss, *J. Mol. Catal. B* **1997**, *3*, 131–138; d) for a review of acylating agents in biocatalysis, see: U. Hanefeld, *Org. Biomol. Chem.* **2003**, *1*, 2405–2415.

[11] One exception is the protease-catalyzed synthesis of carbohydrate-amino acid conjugates with amino acid vinyl esters: V. Boyer, M. Stanchev, A. J. Fairbanks, B. G. Davis, *Chem. Commun.* **2001**, 1908–1909. Vinyl esters of protected amino acids have been prepared by a formal transesterification with vinyl acetate using $PdCl_2$/NaCl: L. J. Loeffler, Z. Sajadi, I. H. Hall, *J. Med. Chem.* **1977**, *20*, 1578–1584.

[12] a) T.-a. Mitsudo, Y. Hori, Y. Watanabe, *J. Org. Chem.* **1985**, *50*, 1566–1568; b) T.-a. Mitsudo, Y. Hori, Y. Yamakawa, Y. Watanabe, *Tetrahedron Lett.* **1986**, *27*, 2125–2126; c) T.-a. Mitsudo, Y. Hori, Y. Yamakawa, Y. Watanabe, *J. Org. Chem.* **1987**, *52*, 2230–2239.

[13] a) C. Ruppin, P. H. Dixneuf, *Tetrahedron Lett.* **1986**, *27*, 6323–6324; b) H. Doucet, J. Hofer, C. Bruneau, P. H. Dixneuf, *J. Chem. Soc. Chem. Commun.* **1993**, 850–851; c) H. Doucet, B. Martin-Vaca, C. Bruneau, P. H. Dixneuf, *J. Org. Chem.* **1995**, *60*, 7247–7255; d) H. Doucet, N. Derrien, Z. Kabouche, C. Bruneau, P. H. Dixneuf, *J. Organomet. Chem.* **1997**, *551*, 151–157.

[14] L. J. Gooßen, J. Petzold, D. Koley, *Chem. Commun.* **2003**, 706–707.

[15] For the use of other metals as catalysts in the alkyne addition, see: Rh: a) C. Bianchini, A. Meli, M. Peruzzini, F. Zanobni, C. Bruneau, P. H. Dixneuf, *Organometallics* **1990**, *9*, 1155–1160; b) A. Lumbroso, N. R. Vautravers, B. Breit, *Org. Lett.* **2010**, *12*, 5498–5501; Re: c) R. Hua, X. Tian, *J. Org. Chem.* **2004**, *69*, 5782–5784; Ir: d) H. Nakagwa, Y. Okimoto, S. Sakaguchi, Y. Ishii, *Tetrahedron Lett.* **2003**, *44*, 103–106; Au: e) B. C. Chary, S. Kim, *J. Org. Chem.* **2010**, *75*, 7928–7931.

[16] General reviews for the metal-catalyzed addition of carboxylic acids to alkynes: a) L. J. Gooßen, N. Rodriguez, K. Gooßen, *Angew. Chem.* **2008**, *120*, 3144–3164; *Angew. Chem. Int. Ed.* **2008**, *47*, 3100–3120; b) C. Bruneau, P. H. Dixneuf, *Angew. Chem.* **2006**, *118*, 2232–2260; *Angew. Chem. Int. Ed.* **2006**, *45*, 2176–2203; c) M. Beller, J. Seayad, A. Tillack, H. Jiao, *Angew. Chem.* **2004**, *116*, 3448–3479; *Angew. Chem. Int. Ed.* **2004**, *43*, 3368–3398; d) C. Bruneau, P. H. Dixneuf, *Chem. Commun.* **1997**, 507–512.

[17] Synthesis of isopropenyl esters of *N*-protected amino acids *via* Ru-catalyzed addition of propyne and aminolysis: a) C. Ruppin, P. H. Dixneuf, S. Lecolier, *Tetrahedron Lett.* **1988**, *29*, 5365–5368; and synthesis of dipeptides by KCN-catalyzed reaction with amino acid esters: b) Z. Kabouche, C. Bruneau, P. H. Dixneuf, *Tetrahedron Lett.* **1991**, *32*, 5359–5362.

[18] N. L. Benoiton, Y. C. Lee, R. Steinaur, F. M. F. Chen, *Int. J. Peptide Protein Res.* **1992**, *40*, 559–566.

[19] We observed higher conversions and selectivities when using isolated Ru complexes instead of their *in situ* prepared counterparts. Therefore, we used the *in situ* method only for the initial ligand screening. In all other cases, isolated Ru complexes were used as catalysts.

[20] The synthesis of the related (−)-DIOP-Ru complex and its use in asymmetric hydrogenation has been described: J. P. Genêt, S. Mallart, C. Pinel, S. Juge, J. A. Laffitte, *Tetrahedron: Asymmetry* **1991**, *2*, 43–46.

[21] Recently, a strong solvent influence on the regiochemistry in Ru-catalyzed addition of acids on alkynes has been reported: C. S. Yi, R. Gao, *Organometallics* **2009**, *28*, 6585–6592.

[22] In certain cases we observed small amounts of oligomers, which resulted from a multiple alkyne addition with amide nucleophiles. This reaction has been reported before, see: L. J. Gooßen, K. S. M. Salih, M. Blanchot, *Angew. Chem.* **2008**, *120*, 8620–8623; *Angew. Chem. Int. Ed.* **2008**, *47*, 8492–8495.

[23] See for example: K. Taguchi, F. H. Westheimer, *J. Org. Chem.* **1971**, *36*, 1570–1572.