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An artificial ruthenium-containing -barrel protein for alkene-alkyne coupling reaction

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**A modified Cp\*Ru complex, equipped with a maleimide group, was covalently attached to a cysteine of an engineered variant of *Ferric hydroxamte uptake protein component: A* (FhuA). This synthetic metalloprotein catalyzed the intermolecular alkene-alkyne coupling of 3-butenol with 5-hexynenitrile. When compared with the protein-free Cp\*Ru catalyst, the biohybrid catalyst produced the linear product with higher regioselectivity.**

Carbon-carbon bond formation is one of the most important reactions in organic chemistry. Therefore, the development of new methodologies and catalysts to form such bonds selectively is an ongoing effort in synthetic chemistry. One promising class of catalysts are the so-called artificial enzymes or biohybrid catalysts (BHCs).1-9 A BHCs consists of a protein scaffold and a non-natural metal center serving as the catalytic site. Thereby, the broad reaction scope of chemical catalysis and the extraordinary selectivity found for enzymes could be combined. This powerful combination might then allow to perform non-natural reactions in a selective manner, like carbon-carbon bond formations.

Carbon-carbon bond-forming reactions catalyzed by artificial metalloproteins contain, *e.g.*, Pd-based catalysts (Suzuki cross-coupling10-12 and allylic alkylations13), Cp\*Rh(III) (benzannulation reactions14-17), Grubbs-Hoveyda type Ru catalyst (olefin metathesis18-29), Cu(II) complexes (Diels-Alder reaction30-36), Rh(I)/CpRh(I) (polymerization of phenyl acetylene37-40 and cylcopropan­ation41, 42), CpCo(I) (trimerization reaction of phenyl acetylene43), and others.1 In an effort to widen the repertoire of such BHCs for bioorthogonal carbon-carbon bond formations, the (5-pentamethylcyclo­pentadienyl)ruthenium(II) (Cp\*Ru) catalyzed alkene-alkyne coupling (an Alder-ene reaction) attracted our attention (Scheme 1).44-46 The intermolecular reaction involves a terminal alkyne and alkene. As products, branched or linear 1,4-dienes are obtained. Additionally, an intramolecular version of this reaction is reported, where the alkene and alkyne are part of one precursor and results in a cyclic product. The general applicability of this reaction in combination with a high functional group tolerance has been shown by Trost and coworkers in various studies ranging from the conversion of benchmark substrates up to the synthesis of natural compounds.44, 47-50

As outlined in Scheme 1, both linear and branched products can be obtained in this reaction. This reaction is believed to proceed through a ruthenacylcopent-2-ene intermediate.44, 46 This intermediate is formed by oxidative coupling of the alkene and alkyne ligand coordinated at the Ru(II) center. The relative orientation of the coordinated alkene and alkyne determines the regioselectivity. It remains difficult to control the regioselectivity, since many factors influence the product distribution (*e.g.*, the nature of the substituents at the alkene and alkyne, solvents, and reaction temperature).44, 45 Influencing the selectivity through a second coordination sphere has not been reported yet.

Here, we report the construction of an artificial metalloprotein capable of catalyzing the alkene-alkyne coupling reaction. A modified Cp\*Ru catalyst was equipped with a maleimide moiety for protein conjugation. As protein scaffold, the previously reported engineered variant of transmembrane protein *Ferric hydroxamate protein component: A* (FhuA)



**Scheme 1.** General reaction scheme of the Cp\*Ru catalyzed alkene-alkyne coupling.



**Scheme 2.** Synthesis of Cp\*Ru complexes **3** and **4** as well as their conjugation to FhuA and subsequent refolding (exchange of sodium dodecyl sulfate (SDS) with MPD) yielding biohybrid catalyst **6**.

(FhuA 1-159\_C545\_V548\_F501\_tev or FhuA ΔCVFtev) was used which contains a cysteine residue in position 96 for anchoring as well as cleavage sites for TEV protease.21 The advantage of this robust -barrel protein lies in its stability towards co-solvents and temperature.51 Furthermore, the protein is capable of fully surrounding the metal cofactor, thereby allowing to study the influence of the second coordination sphere on the alkene-alkyne coupling reaction. 5-Hexynenitrile and 3-butenol were chosen as substrates, due to their (partial) water solubility. To equip a Cp\*Ru complex with a maleimide moiety, the previously reported sandwich complex [(5-C5Me4CH2OH)Ru(6-C10H8)]BF4 (**1**) bearing an hydroxymethyl function at the tetramethylcyclopentadienyl ligand was used as the starting complex.52 The alcohol function was connected to the maleimide group by esterification with 3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanoyl chloride (**2**), yielding Cp\*Ru complex **3**. Subsequently, the naphthalene ligand was exchanged by acetonitrile to give pre-catalyst **4** (Scheme 2).

Ligand exchange was necessary because complex **3** did not convert the substrates at all (Table 1, entry 1). The strong coordination of the naphthalene ligand apparently saturates the Ru(II) center and prevents coordination of the substrates. Complexes **3** and **4** were isolated and characterized through NMR spectroscopy as well as high resolution ESI-MS. The presence of the maleimide moiety in complexes **3** and **4** was confirmed by the characteristic singlet of the maleimide double bond protons at around ** = 6.7 ppm in their 1H NMR spectrum (Figure S1 and S5, respectively).

Next, we performed conjugation of complex **4** to FhuA (Scheme 2b). Under basic conditions (pH = 8), an excess of the respective complex was incubated with the protein. Afterwards, the excess of catalyst was removed through exhaustive washing with acetonitrile and the -barrel structure protein was renatured through dialysis. Structural integrity was confirmed by CD spectroscopy. The CD spectrum of the BHC **6** revealed characteristic bands for a -barrel structure with a minimum at **max = 215 nm and a maximum at **min = 196 nm (Figure 1).21, 51 CD spectroscopy at variable temperatures confirmed structural integrity up to 60 °C. Above 60 °C, minimal changes in the CD spectrum were observed and the protein was not fully denatured below 90 °C (Figure S9). Structural integrity was further confirmed in the presence of MeCN (Figure S10). Successful catalyst conjugation was indicated by fluorescence titration using ThioGlo® as fluorescence dye. Fluorescence titration showed nearly quantitative conjugation of the catalyst to the protein scaffold (Figure S11).

As the molecular weight of FhuA is too high to analyze with mass spectrometry, a TEV-protease was used to digest the protein site-selectively.21 The introduced TEV-cleavage sites are located in the loops of sheets 7 and 8, releasing upon digestion a 5.9 kDa fragment that contains the cysteine residue with the catalyst is attached. This fragment was subsequently analyzed by MALDI-ToF-MS (Figure S12). The observed mass is in good agreement with the attached ruthenium catalyst **4**, albeit a MeCN ligand had been replaced by water (calculated mass: 6432 Da; observed mass: 6421 Da).

The characterized molecular catalyst **4** as well as the biohybrid catalyst **6** were next probed in the alkene-alkyne coupling



**Figure 1.** Variable-temperature CD spectra of **6**. The CD spectrum of FhuA shows a characteristic minimum at  = 218 nm and a maximum at  = 196 nm, indicating a -sheet structure.51 Structural integrity is confirmed for temperatures up to 60 °C. Above ca. 60 °C, minimal changes in the CD spectra are observed, however, the protein is not fully denatured up to 90 °C.

**Table 1.** Results of the alkene-alkyne coupling reaction of 3-butenol (**7**) with 5-hexynenitrile (**8**) catalyzed by Cp\*Ru-based catalysts.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | | | | | | |
| Entry*a* | [Ru] | Conv.*f* [%] | TON | **9**B:**9**L*f* | TOF [h-1] |
| 1 | **3** | 0 | - | - | - |
| 2 | **4***b* | 62 | 12 | 1:1.6 | 0.5 |
| 3 | **4***c* | 56 | 11 | 1:3.8 | 0.5 |
| 4 | **4** | 70 | 14 | 1:1.6 | 0.6 |
| 5 | -*b* | 0 | - | - | - |
| 6 | -*c* | 0 | - | - | - |
| 7 | - | 0 | - | - | - |
| 8 | FhuA | 0 | - | - | - |
| 9 | **3**@FhuA*d* | 0 | - | - | - |
| 10 | **6***d* | 76 | 152 | 1:16 | 6.3 |
| 11 | **6***d*,*e* | 85 | 170 | 1:16 | 3.5 |
| *a* Reaction conditions: Aqueous buffer solution (50 mM MPD, 100 mM NaPi, pH = 8, 1 mM EDTA), 20 (v/v)% MeCN, 5 mol% catalyst. *b* pH = 6. c pH = 7. d 0.5 mol% catalyst. e Reaction time 48 h. f Determined by GC MS. Error on conversion ±4%. | | | | | | |

reaction of 3-butenol (**7**) with 5-hexynenitrile (**8**) in aqueous solutions (Table 1). Unlike the naphthalene complex **3** (Table 1, entry 1), the tris(acetonitrile) complex **4** catalyzed the alkene-alkyne coupling reaction. In the tested range from pH = 6 to 8 (Table 1, entries 2-4), the conversion was highest at lower pH values with a branched to linear ratio (B:L) of about one (Table 1, entry 4), whereas the B:L ratio was lower at pH = 7 (Table 1, entry 3). The branched product is favored in organic solvents, as reported by Trost and coworkers for a similar catalyst ([(5-cyclopentadienyl)ruthenium(II)(MeCN)3][PF6]). The use of an aqueous reaction environment apparently changes the preference for the branched product. Without a catalyst present, the alkene-alkyne coupling does not occur, regardless of the pH value of the solution (Table 1, entries 5-7). In the presence of the apo-protein FhuA (without Ru-catalyst attached), product formation was not observed (Table 1, entry 8). The same applies to complex **3** when bound to FhuA (Table 1, entry 9). Interestingly, FhuA significantly influenced the behavior of complex **4** when conjugated to FhuA (BHC **6**). The BHC **6** shows an increased activity compared to the protein-free catalyst **4** (TON: 152 vs. 14; TOF: 6.3 h-1 vs. 0.6 h-1; Table 1, entries 10 and 4). Moreover, the B:L ratio is markedly influenced upon conjugation to the protein in favor of the linear product (Table 1, entry 10). Extension of the reaction time from 24 h to 48 h had only minor effect on the reaction (Table 1, entry 11). Nonetheless, the B:L ratio remained unchanged.

Conclusions

In summary, we report here a new BHC based on a variant of the transmembrane -barrel protein FhuA equipped with the modified (5-pentamethylcyclopentadienyl)ruthenium(II) catalyst **4**. The resulting BHC **6** was characterized by fluorescence titration, CD spectroscopy, and MALDI-ToF MS. In the alkene-alkyne reaction of 3-butenol (**7**) with 5-hexynenitrile (**8**), the BHC **6** showed a 10-fold increase in activity and preferred formation of the linear over the branched product (B:L = 1:16).

Compared to our previously synthesized BHC containing a modified (5-cyclopentadienyl)rhodium(I) catalyst for the polymerization of phenylacetylene53 or a (5-cyclopentadienyl)­cobalt(I) catalyst for the cyclotrimerization of phenyl­acetylene,43 the length of the linker seems to have an influence on the regioselectivity of the corresponding reaction. With the longest linking unit attached to the CpCo-catalyst (eight atoms between the cyclopentadiene and the maleimide moiety), no effect on the product distribution was observed. In the study presented here (five atoms chain length) as well as in the study with the CpRh-catalyst (two atoms chain length), the corresponding C-C bond forming reactions were influenced in their regioselectivity. Based on the mechanism proposed by Trost *et al.* (Scheme 3), we assume that during the oxidative addition step the orientation of the alkyne substrate (**IIa** or **IIb**) is affected by the interaction with the amino acid residues surrounding the ruthenium site. The apparently somewhat counter-intuitive preference of **IIa** leading to the linear product *via* **IIIa** and **IVa** may be due to some weak interaction of the polar substituents of the substrate molecules.



**Scheme 3.** Proposed catalytic cycle for the alkene-alkyne coupling reaction. Adapted from Trost *et al.*[46]

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Conflicts of interest

There are no conflicts to declare.

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