ChemPubSoc Europe

DOI: 10.1002/chem.201502381

CHEMISTRY A European Journal Full Paper

Metathesis

Ring-Closing and Cross-Metathesis with Artificial Metalloenzymes Created by Covalent Active Site-Directed Hybridization of a Lipase

Manuel Basauri-Molina, Dide G. A. Verhoeven, Arnoldus J. van Schaik, Henk Kleijn, and Robertus J. M. Klein Gebbink*[a]

Abstract: A series of Grubbs-type catalysts that contain lipase-inhibiting phosphoester functionalities have been synthesized and reacted with the lipase cutinase, which leads to artificial metalloenzymes for olefin metathesis. The resulting hybrids comprise the organometallic fragment that is covalently bound to the active amino acid residue of the enzyme host in an orthogonal orientation. Differences in reactivity as well as accessibility of the active site by the functionalized inhibitor became evident through variation of the anchoring motif and substituents on the N-heterocyclic carbene ligand. Such observations led to the design of a hybrid that is active in the ring-closing metathesis and the cross-metathesis of *N,N*-diallyl-*p*-toluenesulfonamide and allylbenzene, respectively, the latter being the first example of its kind in the field of artificial metalloenzymes.

Introduction

The development of semi-synthetic metal–protein hybrids through the combination of transition-metal catalysts and protein scaffolds represents a bioinspired approach to catalyst design. Such hydrides may not only display enhanced catalytic activities, but also show enhanced catalytic selectivities as a result of a second coordination sphere that is created around the metallic center by positioning it within the protein host.[1] A handful of hybridization strategies have been developed towards the formation of these conjugates, which include: supramolecular anchoring of metallated biotin motifs in streptavidin,[2] alkylation of cysteine and lysine residues with metallated electrophilic ligands,[3] cofactor reconstitution,[4] embedding of nanoparticles in proteins,[5] protein biosynthesis with artificial amino acids,[6] and active site functionalization of enzymes.[7–8]

With an interest in the development of hybridization strategies and the structural design of metalloproteins, our group has reported a method for the active-site-directed covalent anchoring of organometallic catalysts in lipases.[9–10] A phosphonate ester that is functionalized with an organometallic fragment acts as a suicide inhibitor of the enzymatic family of serine hydrolases and forms a covalent, irreversible bond with the serine residue of the catalytic triad. This method links the metallic center to the former active amino acid residue of the enzyme, which is the location with greater potential for generation of a second coordination sphere. Moreover, the activity of phosphonate esters is not limited to a single lipase; hence, the choice of different lipases is an alternative strategy to the use of mutagenic techniques for the screening of protein scaffolds. Furthermore, catalyst leaching is prevented by the covalent binding constitution.

Following this method, we reported the successful enhancement of the chemoselectivity that is exerted by a Rh(NHC) hydrogenation catalyst (NHC=N-heterocyclic carbene) through its covalent anchoring in the active site of the lipases cutinase and *Candida antarctica* lipase B (CalB) by a synthetic phosphonate–NHC cofactor.[11] Therein, the location of the active site in CalB was deeper than that of cutinase, which resulted in a larger extent of sterical influence of the enzyme host over the metallocatalyst. In continuation of this research, we envisioned the coordination of the designed phosphonate–NHC cofactor with other metal centers to explore different catalytic reactions with the corresponding semi-synthetic enzyme hybrids, which is reported herein.

In the field of artificial metalloenzymes, there is a specific interest in nonbiological, catalytic applications because these extend the reactivity repertoire of enzymes, for example in C–C coupling reactions, which includes allylic alkylation[12] and Diels Alder cyclizations.[13] Grubbs-type olefin metathesis catalysts recently proved to be compatible with proteins as substrates (in vitro).[14] This prompted the incorporation of Grubbs-type complexes, based on Ru(NHC)s, in protein structures to assess the potential promotion of selectivity that

[a] *Dr. M. Basauri-Molina, D. G. A. Verhoeven, A. J. van Schaik, H. Kleijn, Prof. Dr. R. J. M. Klein Gebbink*
Organic Chemistry and Catalysis
Institute for Nanomaterials Science, Faculty of Science
Utrecht University, Universiteitsweg 99
Utrecht 3584CG (The Netherlands)
E-mail: r.j.m.kleingebbink@uu.nl

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201502381.

is conferred by the aforementioned generation of a sterically demanding environment.[15]

Along this line, a number of artificial metalloenzymes for olefin metathesis have been created.[8,16–18] Ward and Hilvert reported the alkylation of a cysteine residue of a small heat-shock-protein variant with a Grubbs catalyst, which contained an electrophilic haloacetamide group; the resultant covalent hybrid can perform the ring-closing metathesis (RCM) reaction of *N,N*-tosyl diallylamine (TDA).[16] Ward and co-workers performed the same reaction with different metalloenzymes, which were formed by the site-specific supramolecular anchoring of a biotinylated Grubbs catalyst in streptavidin.[18a] They further optimized the structure of the hybrids to create an orthogonal biotinylation of the Grubbs catalyst, and they extended the RCM scope to synthesize a coumarin derivative.[18b] Following the alkylation of a cysteine residue, Schwaneberg and Okuda incorporated the catalyst in a variant of the β-barrel protein FhuA and performed ring-opening metathesis polymerization (ROMP) of an oxanorbornene derivative.[17a] Differences in catalytic activities and a slight influence over *E/Z* product selectivity were found when using cofactors of different length.[17b] Hirota and co-workers used haloacetamide-functionalized Grubbs catalysts and positioned these in the active site of the protease α-chymotrypsin to carry out the RCM reaction of TDA and other polar and apolar diallylamines.[8] These efforts highlight the current interest in the development of semi-synthetic metalloenzymes for olefin metathesis.

Herein, we have synthesized and studied a series of new Ru(NHC)–protein hybrid complexes, which have been produced by the active-site-directed method by using Grubbs catalysts with orthogonally positioned phosphonate-containing lipase inhibitors (Figure 1). The bulkiness and tether length of the tailored NHC ligands influenced both the hybridization feasibility and the catalytic activity. These semi-synthetic metalloenzymes for olefin metathesis proved active in the ring-closing metathesis of TDA as well as the cross-metathesis of allylbenzene, which is a reaction that has not yet been explored with artificial enzymes.

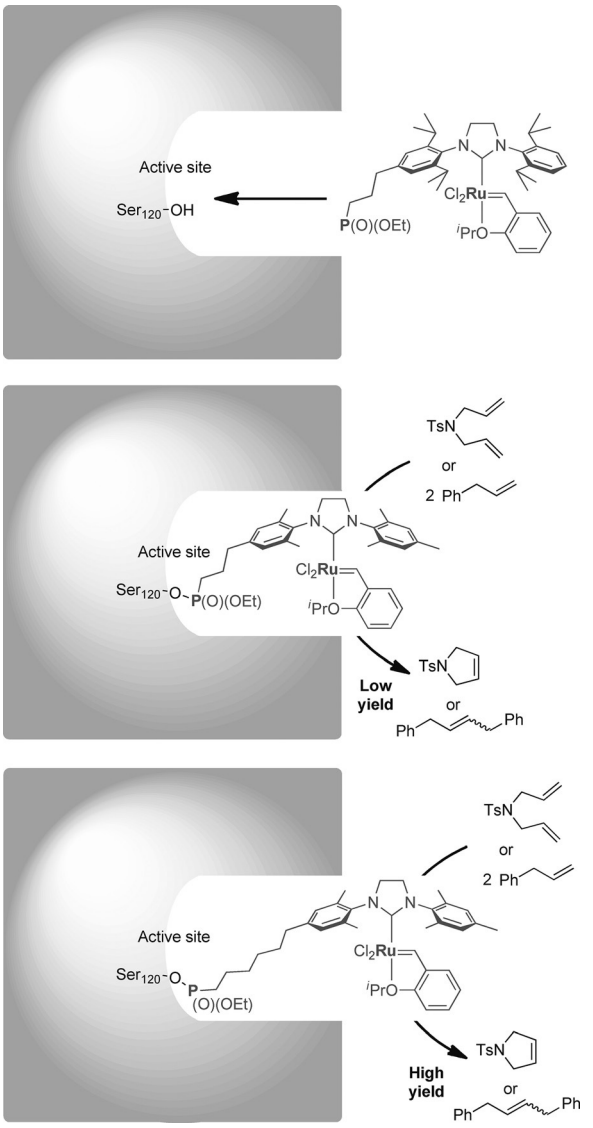


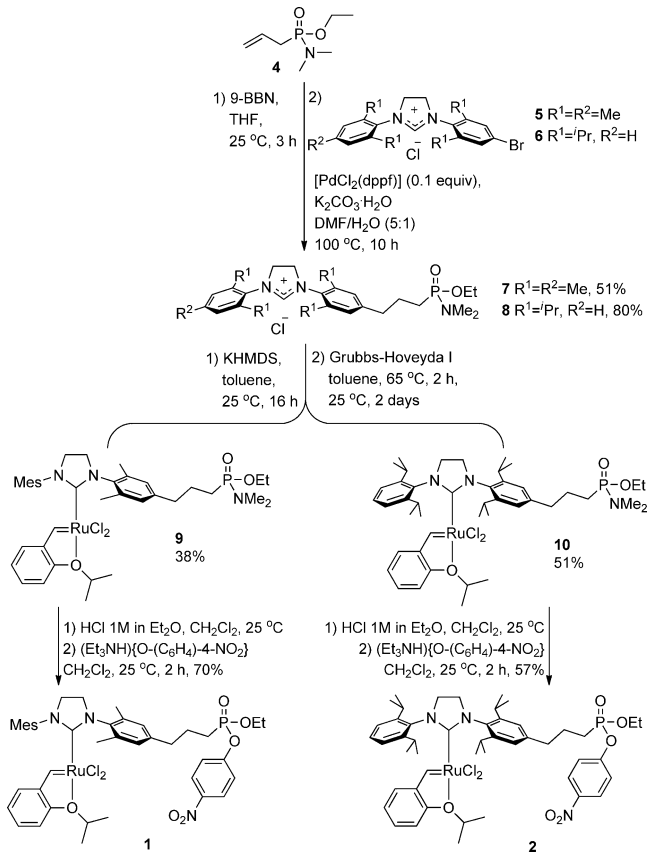
Figure 1. Structural variations of Grubbs-like lipase inhibitors lead to differences in the hybridization and catalytic activity of the targeted active-site-directed covalent artificial metalloenzymes for olefin metathesis.

Results and Discussion

The short-tethered SIMes-containing (SIMes = 1,3-dimesityl-4,5-dihydroimidazolinium) preligand **7**[11] was used as the building block in the construction of the lipase inhibitor **1** (Scheme 1). The Hoveyda chelate (*o*-propoxyphenyl-methylylidene)[19] was chosen as the benzylidene ligand because of its wide use in aqueous olefin metathesis. In parallel, we designed and synthesized a ligand with a slightly more protecting behavior towards the ruthenium center. To this end, we substituted the trimethylphenyl N-substituents for the bulkier diisopropylphenyl groups to give preligand **8** and, consequently, inhibitor **2** (Scheme 1).

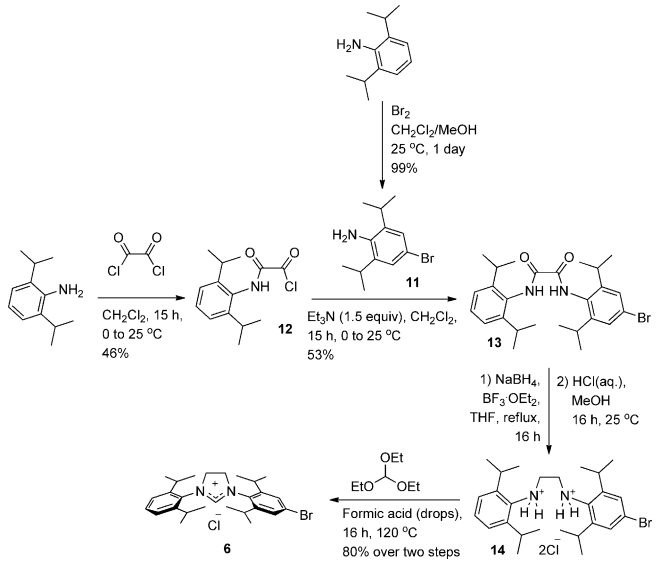
Preligand **7** was synthesized through the Suzuki cross-coupling of hydroboronated ethyl *N,N*-dimethyl allyl phosphonamidate **4** and 1-mesityl-3-(4-bromo-2,5-dimethylphenyl)-4,5-dihydroimidazolinium chloride **5**. Metallation to give the ruthenium complex **9** was achieved by initial deprotonation of the imidazolinium salt **7** with the non-nucleophilic base KHMDS to avoid nucleophilic attack on the phosphorous center; the free carbene was then directly metallated with Grubbs–Hoveyda I precursor by using a low concentration of reagents at room temperature over a long reaction time of 2 days. Under more harsh conditions, it was noticed that intra- or intermolecular interactions occurred. The purified product **9** still showed a limited lifetime of 5 days under inert conditions in the presence of residual solvent; therefore, it was used for the next reaction directly after purification (see below).

Hartwig has reported that Grubbs catalysts with isopropyl NHC substituents showed increased resistance to aqueous conditions in the presence of an enzyme compared with the corresponding catalyst with mesityl substituents.[14d] Accordingly, complex **10** was designed, for which the ligand precursor, 1-(2,6-diisopropylphenyl)-3-(4-bromo-2,6-diisopropylphenyl)-4,5-dihydroimidazolinium salt **6** was synthesized by the



Scheme 1. Synthesis of Grubbs–phosphonate-based lipase inhibitors **1** and **2** (9-BBN = 9-borabicyclo[3.3.1]nonane, dppf = bis(diphenylphosphino)-ferrocene).

bromination of 2,6-diisopropylaniline followed by its coupling with chloro oxallyl anilide **12** (Scheme 2). The resulting diamide **13** was reduced by using a combination of sodium borohydride and trifluoroborane, and the subsequent protonation followed by heating at reflux with triethyl orthoformate led to cyclization to give the desired ligand precursor **6**. Afterwards, the synthesis of preligand **8** was conducted in a similar manner as that of **7** (see above). Likewise, the metallation procedure that we had developed for the formation of ruthenium complex **9** was also successful here to give the complex **10** (Scheme 1), which showed improved stability with no changes in either the color or the ^{1}H NMR spectrum observed upon storage. The final step for the synthesis of inhibitors **1** and **2** involved the elimination of dimethylamine by using hydrogen chloride and then substitution of the resulting halide with *p*-nitrophenolate (pNP).



Scheme 2. Synthesis of brominated imidazolinium chloride **6**.

Interestingly, the metallation of the carbene towards both complexes **9** and **10** resulted in a mixture of isomers, in which the Hoveyda ligand lies in a plane with the benzylidene in the direction of either the substituted or the non-substituted mesityl group. According to the ^{1}H NMR spectrum of the former moiety, the ratio of these isomers was 72:28 and 74:26 for complexes **9** and **10**, respectively. However, for both inhibitors **1** and **2**, only one isomer was observed (see the Supporting Information for detailed spectra of organometallic species).

Cutinase was chosen as the host enzyme because of its reactivity with hydrophobic substrates, its relatively exposed active site,[20] and its capability to induce steric hindrance in the artificial metalloenzymes.[11] Firstly, a reaction to modify cutinase with inhibitors **1** and **2** was carried out with an excess of complex **1** in accordance with our previously reported biphasic conditions for a Rh(NHC) inhibitor[11] (Tris-HCl buffer pH 8.5 with CH_2Cl_2, 5% *v/v* as co-solvent), which resulted in the successful formation of hybrid **Ru1–cut**. The excess of complex **1** was eliminated by separation of water-insoluble species and dialysis of the hybrid; the covalent constitution of the hybrid allowed for the latter procedure without disintegration. ESI-MS of a dialyzed and denatured sample of **Ru1–cut** showed the desired conjugate with incorporation of the Grubbs complex in a 1:1 stoichiometry (Figure 2, top). This result gives evidence for the irreversible nature of the hybridization at the former active site.

On the other hand, when cutinase was treated with inhibitor **2**, the formation of the desired hybrid was not achieved even after several attempts that used different reaction times and co-solvents. We attributed the latter observation to the bulkier character of the N-substituents of the inhibitor, in which the isopropyl moieties possess a larger steric demand than the methyl groups.[21] The difference between the inhibition of cutinase with complexes **1** and **2** suggests a fine sensitivity of cutinase towards these potential inhibitors. Interestingly, when the reaction to incorporate complex **2** was carried out over several days at room temperature with DMSO as the co-solvent, we only observed incorporation of the ligand within the enzyme without the ruthenium center (Figure 2, bottom, hybrid **L2–cut**). This may originate from a slow decomposition of the Ru–NHC complex in the reaction medium, which allows cutinase to react with the remaining ligand. This indicated that the unsuccessful formation of **Ru2–cut** was due to the hindrance of the complete inhibitor structure and not only the bulk of the bis(isopropyl)phenyl groups of the NHC ligand. The

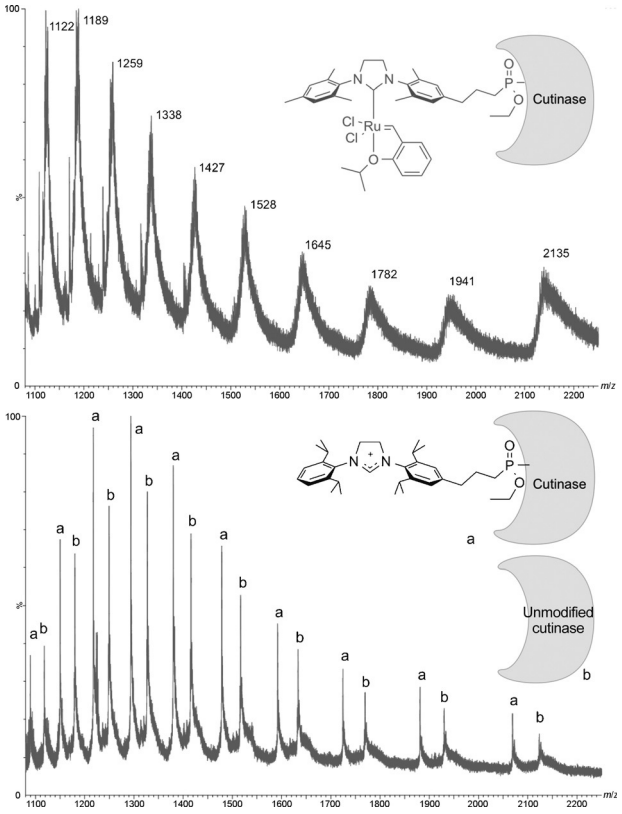
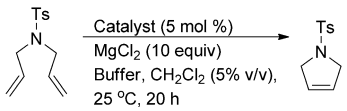


Figure 2. ESI-MS spectra of **Ru1–cut** (top) and **L2–cut** (bottom), the latter as a result of the reaction of cutinase with complex **2** for prolonged times.

latter insight led us to design a new Grubbs inhibitor (see below).

For the evaluation of the catalytic activity of the new hybrid **Ru1-cut** catalyst in an RCM reaction, we considered TDA as a suitable substrate because of its recurrent use in olefin metathesis with artificial enzymes.[8,16,18] Initially, a reaction was carried out with **Ru1–cut** (5 mol%) in Tris-HCl buffer at pH 8.5, CH_2Cl_2 (5% *v/v*), an excess of $MgCl_2$ as a source of chloride,[22] and under constant stirring to promote substrate–enzyme interaction, but this resulted in no conversion at room temperature after 40 h (Table 1, entry 1). Previous reports mention a negative effect of basic pH on the catalytic performance of semi-synthetic hybrids.[16,17b,18a] In addition, a histidine side chain on the enzyme may coordinate to the ruthenium center and inhibit its activity.[23] Therefore, we dialyzed the hybrid catalyst **Ru1–cut** and tested it in aqueous hydrochloric acid at pH 2, but catalysis was still unsuccessful (entry 2); instead, precipitation of the protein content was observed, which was attributed to denaturation of the cutinase at this very low pH. Under less acidic conditions (pH 5), native cutinase has demonstrated both stability and activity, albeit with lower rates.[24] In view of all this, we prepared and tested the hybrid catalyst **Ru1–cut** at pH 5 in acetate buffer; after 2 days, the hybridization reaction led to the successful formation of the hybrid **Ru1–cut**, which was stored at pH 5. In spite of these changes, the RCM reaction gave the desired 1-tosyl-2,5-dihydropyrrole in a low yield of only 1% (entry 3). The commercial

Table 1. Catalytic evaluation of hybrids **Ru1–cut** and **Ru3–cut** for the ring-closing metathesis of *N,N*-diallyl *p*-toluenesulfonamide.



Entry	Catalyst	Media[a]	Yield [%]
1	**Ru1–cut**	Buffer pH 8.5[b,c]	–
2	**Ru1–cut**	HCl 0.01 M	–
3	**Ru1–cut**	Buffer pH 5	1
4	Grubbs–Hoveyda II	Buffer pH 5+cutinase	90
5	**Ru3–cut**	Buffer pH 8.5[c]	–
6	**Ru3–cut**	Buffer pH 5	84

[a] Tris-HCl buffer was used for pH 8.5 and sodium acetate buffer for pH 5. [b] For 20 and 40 h. [c] With and without $MgCl_2$.

Grubbs–Hoveyda II catalyst was also tested in the presence of unmodified cutinase to determine if there was an undesired incompatibility between these components; however, the reaction took place successfully under these conditions (entry 4).

Interestingly, the high activity of the commercial Grubbs catalyst in the presence of native cutinase compared with the notably lower activity of the **Ru1–cut** hybrid (Table 1, entries 3 and 4) points to little or no risk of leaching of the organometallic fragment from the enzyme. Additionally, treatment of the catalysis reaction mixture, which contains **Ru1–cut**, with an excess of organic solvent to extract the product did not yield the desired dihydropyrrole product. This result indicates that the organometallic fragment is also not released after denaturation. These observations stress the importance of the prior formation and isolation of the hybrid for the evaluation of its catalytic activity to avoid interference of unsupported catalytic material in the medium.

In spite of the well-known solvent accessibility of cutinase's active site,[25] our results pointed to the probable blocking of the metallic center after its embedment in the enzyme host. To provide insight into this, we constructed computational models of the hybrid.[26] By the docking of a Grubbs–Hoveyda II structure that contained the phosphorylated propenyl tail onto cutinase (Figure 3), it was found that the Grubbs catalyst is not entirely embedded in the protein, but that the ruthenium center is surrounded by amino acid residues that are known to partially block the entrance of substrates to the cutinase active site (helical flap comprising of residues 81–85 and a binding loop involving residues 178-186[27]). Moreover, the benzylidene carbon, the labile site for the substrate, is in close proximity to the protein scaffold, which surrounds it from opposite sides (5.2 Å to Leu_{81} and 5.4 Å to Leu_{182}). Therefore, steric hindrance from the protein scaffold is a probable cause of the lack of metathetical reactivity of **Ru1–cut**.

We then designed the new Grubbs-based inhibitor **3**, which contains the larger hexylene tether (Scheme 3). Accordingly, phosphonate precursor **15** was synthesized by alkylation of diethyl phosphite and sequential transformation into a chlorophosphate and the phosphonamidate **16** in situ. Cross-coupling with imidazolinium salt **5** and further metallation

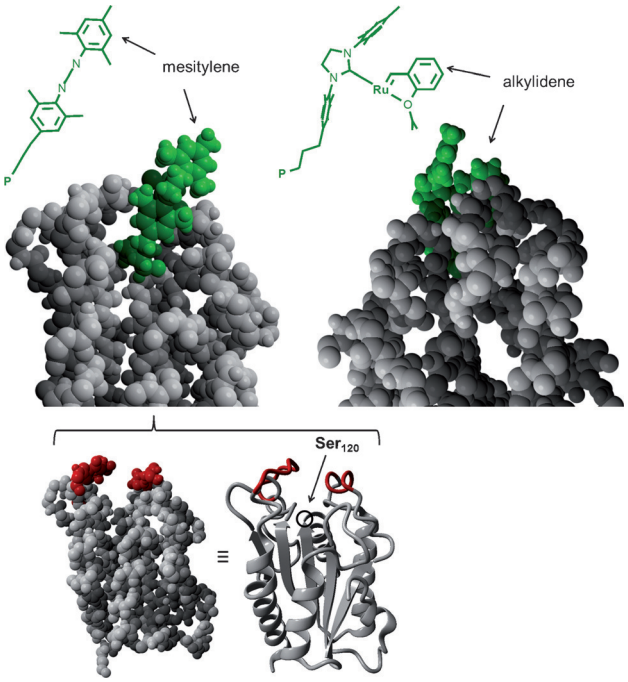
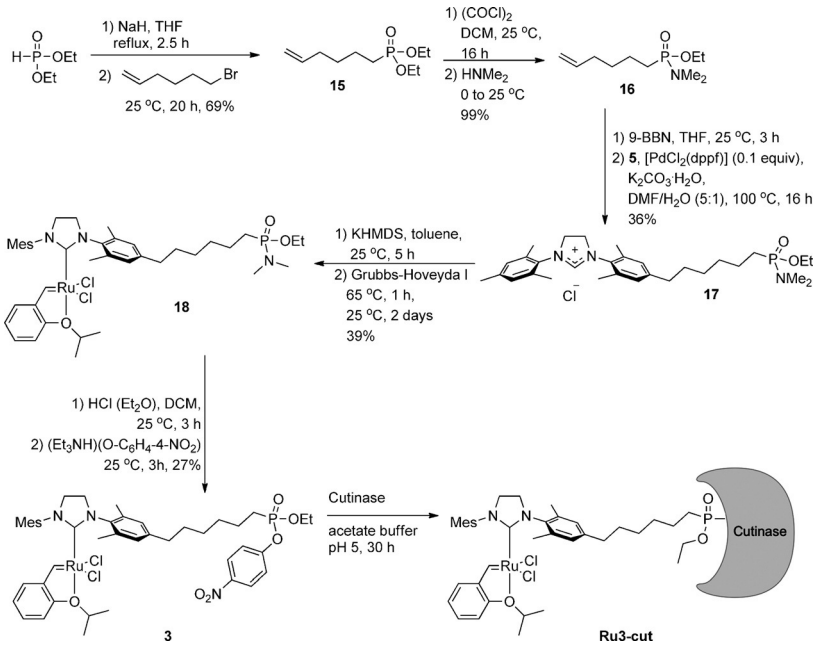


Figure 3. Docking modeling of **Ru1–cut**:[26] rear view (top left), side view (top right), cutinase without the complex (bottom) with the complex in green and the blocking residues in red.



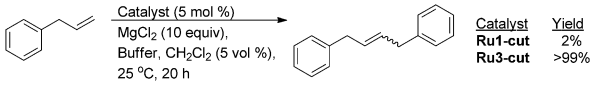
Scheme 3. Synthesis of lipase inhibitor **3** and hybrid **Ru3–cut**.

steps were equivalent to those of the previous inhibitors. A mixture of isomers was again observed after the metallation to give **18** with a ratio of 56:44, which is suggestive of a more homogeneous mixture compared with the ratio of isomers that were observed for complexes **9** and **10**. Substitution of the dimethylamine group gave the inhibitor **3**, which also showed a mixture of isomers with a ratio of 8:2, whereas for complexes **1** and **2** only one isomer was observed (see above). Inhibition of cutinase was carried out at pH 5 for 30 h by using an excess of complex **3**; analysis by ESI-MS after dialysis showed the desired hybrid **Ru3–cut** (see the Supporting Information).

The new hybrid **Ru3–cut** gratifyingly afforded a high conversion towards the dihydropyrrole product in the RCM reaction of TDA at pH 5 (Table 1, entry 6). At basic conditions of pH 8.5 both with or without magnesium chloride, we observed no conversion (entry 5). From these observations, it can be deduced that the new ligand, in combination with the acidic conditions and magnesium chloride, gives rise to a catalytically active olefin metathesis hybrid. Magnesium chloride, as mentioned above, acts as a chloride source, which is important because it has been observed that one chloride ligand bound to the ruthenium center can promote the metathetical reaction[22] ($MgCl_2$ [18a] or KCl[8] have been used with other reported artificial enzymes for olefin metathesis).

Elongation of the cofactor tether successfully allowed for enhanced catalytic activity in the hybrid owing to the reduction of the steric hindrance of protein residues towards substrate approach (see Figure 1). The increment of the aliphatic tether could alternatively confer a degree of flexibility to the inhibitor, which allows for a different directionality of the organometallic fragment with respect to the enzyme pocket. Notably, an influence of the protein scaffold in **Ru3–cut** over the catalytic Ru center is still observed, which leads to a difference in the catalytic yield when compared to the commercial catalyst (Table 1, entries 4 and 6).

Finally, the activity of the two metalloenzymes was tested in the formal cross-metathesis of allylbenzene (Scheme 4). Under the optimized conditions mentioned above (acetate buffer, pH 5, 5 mol% of catalyst, RT, 20 h), the desired product 1,4-diphenylbut-2-ene was formed in a very low yield of 2% by **Ru1-cut** but in quantitative yield by **Ru3-cut**. Product *E*/*Z* ratios of 5:3 for **Ru1-cut** and 4:7 for **Ru3-cut** were found, which are comparable to the values that were afforded by the unsupported Grubbs–Hoveyda II catalyst (*E*/*Z* = 4:9).



Scheme 4. Catalytic evaluation of hybrids **Ru1–cut** and **Ru3–cut** for the cross-metathesis of allylbenzene.

Conclusion

The development of a new hybridization technique represents a fundamental advance in the search for selective and/or versatile semi-synthetic enzymes. In this work, we have made use of a number of favorable criteria that, to the best of our knowledge, were separately documented in previous studies of artificial metalloenzymes for olefin metathesis. Active-site-directed hybridization, covalent metal–enzyme bonding, orthogonal orientation, and cofactor length tuning have all been integrated into the semi-synthetic hybrids that are described herein. Following the covalent inhibition of cutinase with Ru(NHC)–phosphonate inhibitors, the formed hybrids could be purified by dialysis, and they keep their covalent character even after denaturation, as observed by mass spectrometry. This hybridization technique also produces an important interaction with the incorporated Grubbs-type catalyst in the pocket around the original active site of the enzyme.

Successful formation of **Ru1–cut** compared with the unsuccessful formation of the analogue **Ru2–cut** proves that the original hybrid design involved a high degree of sensitivity towards steric effects at the enzyme's active site. The use of the larger but less rigid inhibitor **3** to overcome the poor catalytic activity of **Ru1–cut** was successful by showing a significant gain in catalytic activity, which suggests a sterical demand in the binding pocket of cutinase not only in terms of volume but also in terms of the shape and orientation of the incorporated organometallic fragment. In our experience with cutinase, we had not before observed rejection of a phosphonate inhibitor due to chemical hindrance or a variation in hybrid activity as a function of the cofactor bulkiness. This sets a clear example for the need of rationalized cofactor design, and it states a reference for the sterical limit that cutinase can accommodate in its pocket. As reported recently for rhodium–protein hybrids,[11] the resulting protein scaffold of cutinase has promoted catalytic selectivity, and this could be enhanced by using a different lipase. Nonetheless, changes in the activity as a function of the cofactor is a promising tool for the search of new artificial enzymes by using cutinase because of the abundance of this relatively small enzyme.

The difference in reactivity of the hybrids against an unsupported catalyst in the presence of the enzyme is a simple proof of the difference between metal–protein interactions inside and outside of a lipase even with an enzymatic cavity that is accessible by the solvent, as in cutinase. The trend in reactivity between the metallohybrids in RCM is also reflected in CM: in both reactions the short-tethered hybrid **Ru1–cut** is outperformed by the larger hybrid **Ru3–cut**. This work represents the first example of the versatility of artificial metalloenzymes for mono- and bimolecular olefin metathesis.

Experimental Section

Materials and methods

Chemical precursors were purchased from commercial sources and used without further treatment, unless stated otherwise. Cutinase was obtained from Novonordisk. Et_2O, hexane, and toluene were dried by using an MBraun MB SPS-800 solvent purification system; dichloromethane and tetrahydrofuran (THF) were dried by distillation from $CaCl_2$ and sodium/benzophenone, respectively, and stored over 4 Å molecular sieves. Tris(hydroxymethyl)aminomethane buffer (Tris-HCl) and sodium acetate buffer were prepared in degassed Milli-Q water and stored in Schlenk flasks. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded at 298 K by using a Varian AS 400 MHz NMR spectrometer at 400, 100, and 162 MHz, respectively. Chemical shifts are reported in ppm and referenced against the residual solvent signal. GC measurements were performed by using a PerkinElmer Autosystem XL gas chromatograph equipped with a PerkinElmer Elite-17 column. GC-MS analyses were performed with a PerkinElmer gas chromatograph Clarus 680 equipped with a PE Elite 5 MS column and coupled to a PerkinElmer mass spectrometer Clarus SQ 8T with EI ionization. Electrospray Ionization (ESI-TOF) mass spectra of chemical products were recorded with a Waters LCT Premier XE KE317 Micromass Technologies spectrometer; mass analyses of protein and hybrid products were calculated as $[M]^{n+} = (M+n)/n$). Ultrafiltration dialysis of proteomic samples was performed with Vivaspin 6 tubes, 10,000 MWCO (PEG membrane). All reactions were carried out by using standard Schlenk techniques under inert conditions with an atmosphere of N_2.

Synthesis of imidazolinium preligands

Compounds 4, 5, and 7 were synthesized in accordance with our reported procedures.[11]

4-bromo-2,6-diisopropylaniline 11: The synthesis was adapted from a literature procedure by Chow et al.[28] A solution of Br_2 (0.21 mL, 4.1 mmol) in CH_2Cl_2/MeOH (10 mL, 1:1 v/v) was added to a stirred solution of 2,6-diisopropylaniline (0.74 mL, 3.9 mmol) in CH_2Cl_2/MeOH (20 mL, 1:1 v/v) at room temperature by an addition funnel over 1.5 h. The orange-red solution was stirred for 1 day. Solvents were evaporated, and the resultant red solid was recrystallized from hexane and further purified by column chromatography (CH_2Cl_2) to give a light orange-red solid (0.47 g, 99%). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 10.09$ (bs, NH_2, 2H), 7.35 (s, ArH, 2H), 3.69 (m, $J = 26$ Hz, *i*PrCH, 2H), 1.28 ppm (d, $J = 6.4$ Hz, *i*PrCH$_3$, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 145.0$, 128.0, 123.9, 123.8, 29.0, 24.2; HRMS (EI): *m/z* calcd for $[M]^+$: 255.0623; found: 255.2681.

2-((2,6-diisopropylphenyl)amino)-2-oxoacetyl chloride 12: Oxalyl chloride (38.4 mL, 0.44 mol) was placed into a three-necked round bottom flask, which was equipped with a condenser and an addition funnel, under Schlenk conditions, and it was cooled to 0 °C. A solution of 2,6-diisopropylaniline (7.68 mL, 40.7 mmol) in dry CH_2Cl_2 (60 mL) was placed in the addition funnel and added slowly to the oxalyl chloride solution under a gentle stream of nitrogen through the reflux condenser. Dry CH_2Cl_2 (20 mL) was used to rinse the funnel, and the nitrogen flow was stopped. After the reaction mixture was stirred overnight, the solvents were evaporated. Dry Et_2O (25 mL) was added, and the solids were removed by filtration. The solvent was removed under vacuum, and hexane (60 mL) was added to the residue. The mixture was vigorously stirred before filtration under a nitrogen atmosphere, and the solids were washed with dry CH_2Cl_2. Evaporation of the solvent gave the product **12** (4.94 g, 46%). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.96$ (bs, NH, 1H), 7.36 (t, $J = 7.6$ Hz, ArH, 1H), 7.22 (d, $J = 7.6$ Hz, ArH, 2H), 3.07 (b, 1H), 2.94 (b, *i*PrCH, 2H), 1.24 (d, $J = 7.2$ Hz), 1.21 ppm (d, $J = 6.8$ Hz, *i*PrCH$_3$, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 168.9$, 153.6, 145.7, 129.4, 128.7, 123.9, 29.0, 23.6 ppm.

***N*¹-(4-bromo-2,6-diisopropylphenyl)-*N*²-(2,6-diisopropylphenyl)-oxalamide 13**: A solution of 4-bromo-2,6-dimethylaniline **11** (5.15 g, 19.3 mmol) and Et_3N (2.67 mL, 19.3 mmol) in CH_2Cl_2 (30 mL) was added dropwise to a solution of compound **12** (4.9 g, 19.3 mmol) in dry CH_2Cl_2 (100 mL) at 0 °C. At the end of the addition, another 0.5 equivalents of Et_3N (1.3 mL, 9.7 mmol) was added. The resulting mixture was stirred at 0 °C for 10 min, then at room temperature overnight. The reaction mixture was filtered, and the collected solid was washed with Et_2O and dried under vacuum. The remaining solids were dissolved in CH_2Cl_2 (40 mL), washed with water, dried over $MgSO_4$, and concentrated under vacuum. The residue was precipitated from Et_2O, and the solids were dried under vacuum to give compound **13** as a white solid (5.04 g, 53%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.8$ (bs, 1H, NH), 7.35 (d, $J =$ 8.0 Hz, ArH, 1H), 7.32 (s, BrArH, 2H), 7.21 (d, $J = 7.6$ Hz, ArH, 2H), 3.01 (dsep, $J = 34.4$ Hz, *i*PrH, 4H), 1.21 (d, $J = 3.2$ Hz, *i*PrCH$_3$, 12H), 1.19 ppm (d, $J = 3.2$ Hz, *i*PrCH$_3$, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 159.7$, 159.4, 148.2, 145.7, 129.6, 129.0, 128.9, 127.1, 123.7, 123.1, 29.2, 29.0, 23.5, 23.3 ppm; HRMS (ESI): *m/z* calcd for $[M+H]^+$: 487.1960; found: 489.1948.

***N*¹-(4-Bromo-2,6-diisopropylphenyl)-*N*²-(2,6-diisopropylphenyl)-ethane-1,2-diaminium 14**: $H_3B{\cdot}SMe_2$ complex (4.9 mL, 51.7 mmol) was added slowly to a suspension of the diamide **13** (5.04 g, 10.3 mmol) in toluene (20 mL) at room temperature. The resulting mixture was stirred overnight at 95 °C under a nitrogen atmosphere. After the reaction mixture was cooled to room temperature, $HCl_{(aq)}$ (1 M) was added until the stirred solution was acidified (pH 3), and the resultant mixture was stirred overnight. The precipitate was collected by filtration, washed with Et_2O, and dried under vacuum. The solid was suspended in CH_2Cl_2 (30 mL) and the mixture was cooled to 0 °C. Et_3N (2.9 mL) was added slowly, and the mixture was stirred at room temperature for 2 h. The solvent was removed under vacuum to give a residue, which was purified by silica gel column chromatography (hexanes/ethyl acetate, 8:2) to give compound **14** (80 mg, 2%, because of the low yield, the following product, **6**, was preferably synthesized directly from compound **13**, see below). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.13$ (s, BrArH, 2H), 7.06 (d, $J = 7.6$ Hz, ArH, 2H), 6.82 (t, $J = 15.6$ Hz, ArH, 1H), 3.73 (bs, NH, 2H), 2.96 (q, $J = 18.0$ Hz, NC*H*$_2$CH$_2$N, 2H), 2.89 (dsep, $J =$ 27.2 Hz, *i*PrH, 4H), 2.80 (q, $J = 22$ Hz, NCH$_2$C*H*$_2$N, 2H), 1.27 ppm (d, $J = 6.8$ Hz, *i*PrCH$_3$, 24H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 139.3$, 134.6, 132.4, 129.0, 128.2, 125.8, 125.3, 122.8, 118.5, 111.1, 52.4, 28.0, 22.2 ppm; HRMS (ESI): *m/z* calcd for $[M+H]^+$: 459.2375; found: 459.2405.

***N*¹-(4-Bromo-2,6-diisopropylphenyl)-*N*²-(2,6-diisopropylphenyl)-4,5-dihydro-1 H-imidazol-3-ium chloride 6** (full synthesis from **13**): $BF_3{\cdot}OEt_2$ (2.62 g, 2.28 mL, 0.0185 mol) was added to a suspension of compound **13** (1.00 g, 2.05 mmol) and $NaBH_4$ (0.47 g, 0.012 mol) in THF (25 mL), and the mixture was heated at reflux for 18 h. The reaction mixture was quenched with MeOH (2 mL) and HCl (1 mL, 10 M), and the solvent was removed under vacuum. THF (10 mL) and HCl (1 mL, 4 M) were added, and the resulting mixture was stirred overnight, after which the solvent was evaporated to give an off white solid. Et_2O (100 mL) and aqueous NaOH (150 mL, 1 M) were added, and the biphasic solution was stirred for 10 min to dissolve the solid. The organic phase was separated and the aqueous phase was extracted with Et_2O (2×90 mL). The combined organic phases were dried over $MgSO_4$, and the resultant filtrate was concentrated under vacuum, and the residue was dissolved in triethylorthoformate (10 mL). HCl (0.2 mL, 10 M) was added, and the mixture was heated to 120 °C for 18 h. The mixture was concentrated under vacuum, after which Et_2O (70 mL) was added, and the precipitate was collected by filtration, washing with CH_2Cl_2, to give compound **6** (0.82 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.84$ (s, NCN, 1H), 7.47 (t, $J = 15.2$ Hz, 1H), 7.38 (s, BrArH, 2H), 7.27 (d, $J =$ 7.6 Hz, ArH, 2H), 4.79 (bs, NCH$_2$CH$_2$N, 4H), 2.99 (dsep, $J = 32.8$ Hz, *i*Pr, 4H), 1.38 (d, $J = 6.8$ Hz, *i*PrMe, 6H), 1.38 (d, $J = 6.8$ Hz, *i*PrMe, 6H), 1.26 (d, $J = 6.8$ Hz, *i*PrMe, 6H), 1.26 ppm (d, $J = 6.8$ Hz, *i*PrMe, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 158.8$, 148.4, 146.0, 131.7, 128.5, 125.0, 55.3, 55.1, 53.4, 29.4, 29.3, 25.4, 25.2, 23.7, 23.6 ppm; HRMS (ESI): *m/z* calcd for $[M-Cl^-]^+$: 469.2213; found: 469.2233.

***N*¹-(2,6-Diisopropylphenyl)-*N*²-(4-(3-((dimethylamino)(ethoxy)-phosphoryl)propyl)-2,6-diisopropylphenyl)-4,5-dihydro-1H-imidazol-3-ium chloride 8**: In a glovebox, compound **4** (0.24 g, 1.36 mmol) was dissolved in dry, degassed THF (10 mL). 9-BBN (0.33 g, 1.36 mmol) was dissolved in dry, degassed THF (15 mL) and slowly added to the solution of **4**. The resulting mixture was stirred for 72 h, after which the solvents were removed under vacuum. The product was used directly in the next reaction.

Compound **6** (0.627 g, 1.24 mmol) was dissolved in degassed DMF (10 mL), [$PdCl_2$(dppf)] catalyst (0.091 g, 0.11 mmol) was dissolved in degassed DMF (20 mL), and $K_3PO_4{\cdot}H_2O$ (0.29 g, 1.365 mmol) was dissolved in degassed H_2O (10 mL). The solution of compound **4** and 9-BBN (formed in situ, 1.365 mmol) was added to a Schlenk flask and diluted in degassed DMF (20 mL). While stirring, the solution that contained compound **6** and the [$PdCl_2$(dppf)] suspension were added sequentially. Degassed DMF/water (9:1) was added to obtain a total volume of 100 mL, and then the K_3PO_4 mixture was added. The resultant black mixture was stirred at 100 °C for 16 h and then cooled to room temperature. The mixture was filtered over Celite and washed with DMF (2×35 mL). The solvents were removed under high vacuum, the resultant solid was dissolved in CH_2Cl_2, and the remaining solids were removed by filtration. Owing to the amphiphilic character of the product, this was extracted into an aqueous solution with H_2O (4×150 mL). The aqueous phases were dried under vacuum, and the resultant dark oil was dissolved in CH_2Cl_2 (15 mL). A spoon of activated carbon (Norit) was added; the mixture was stirred for 1 h then filtered over Celite, washing with CH_2Cl_2 (40 mL), and dried in vacuum to give the product **8** (0.6 g, 80%). ^{1}H NMR (400 MHz, CD_2Cl_2): $\delta = 8.17$ (bs, 1H, NCHN), 7.46 (t, $J = 15.6$ Hz, 1H), 7.27 (d, $J = 7.6$, ArH, 2H), 7.06 (s, BrArH, 2H), 4.84 (bs, NCH$_2$CH$_2$N, 2H), 4.13 (m, CH$_2$*C*H$_2$CH$_2$P, 2H), 3.99 (m, CH$_2$CH$_2$*C*H$_2$P, 2H), 3.84 (m, *C*H$_2$CH$_2$CH$_2$P, 2H), 3.03 (dsep, $J = 32.8$ Hz, *i*Pr, 4H), 2.67 (bs, NC*H*$_3$CH$_3$, 3H), 2.64 (bs, NCH$_3$C*H*$_3$, 3H), 1.39 (d, $J =$ 6.8 Hz, *i*PrMe, 6H), 1.28 (d, $J = 7.2$ Hz, OCH$_2$C*H*$_3$, 3H), 1.23 ppm (d, $J = 6.8$ Hz, *i*PrMe, 6H); ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta = 158.1$, 146.2, 145.2, 131.6, 129.2, 127.2, 125.0, 125.0, 59.2, 59.2, 55.3, 55.2, 53.4, 36.7, 36.5, 36.1, 36.1, 29.2, 29.1, 25.4, 25.4, 16.3, 16.2 ppm; ^{31}P NMR (81 MHz, CD_2Cl_2): $\delta = 36.09$ ppm; HRMS (ESI): *m/z* calcd for $[M-Cl^-]^+$: 586.4032; found: 586.4029.

Diethyl hex-5-en-1-ylphosphonate 15: Diethyl phosphite (1.85 mL, 14.37 mmol, 1.28 equiv) was added dropwise by syringe to a suspension of sodium hydride (60% disp. in mineral oil, 0.66 g, 15.15 mmol, 1.35 equiv) in dry THF (6 mL). After the initial gas evolution had ceased, the reaction mixture was stirred for a further 30 min at room temperature followed by heating at reflux for 2.5 h. The reaction mixture was cooled to 0 °C, and 6-bromo-1-hexene (1.5 mL, 11.22 mmol) was added dropwise by syringe. Upon complete addition, the ice bath was removed and the reaction mixture was stirred for 20 h at room temperature. H_2O (50 mL) was added, and the mixture was extracted with CH_2Cl_2 (3× 125 mL). The combined organic phases were washed once with H_2O and concentrated under vacuum. The residue was purified by column chromatography (Et_2O/MeOH, 9:1) to yield compound **15** as a pale yellow oil (1.71 g, 7.79 mmol, 69%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.70$–5.83 (m, 1H), 4.90–5.03 (m, 2H), 3.99–4.15 (m, 4H),

2.01–2.09 (m, 2H), 1.53–1.79 (m, 4H), 1.40–1.51 (m, 2H), 1.30 ppm (t, $J=7$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=138.2$, 114.8, 61.4, 33.2, 26.2, 24.8, 21.9, 16.4 ppm; ^{31}P NMR (81 MHz, $CDCl_3$): $\delta=$ 32.32 ppm.

Ethyl P-(hex-5-en-1-yl)-*N,N*-dimethylphosphonamidate 16: Oxalyl chloride (1.97 mL, 23.29 mmol, 3 equiv) was added dropwise by syringe to a stirred solution of compound **15** (1.71 g, 7.79 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 17 h at room temperature, and then the volatiles were removed under vacuum. This crude material was dissolved in CH_2Cl_2 (10 mL) and added dropwise by syringe to a stirred solution of dimethylamine (33% in absolute ethanol, 27.7 mL, 155.2 mmol, 20 equiv) at 0 °C. Upon complete addition, the mixture was stirred for 5 min, the ice bath was removed, and it was stirred for a further 20 h at room temperature. The reaction mixture was concentrated under vacuum, and the residue was purified by column chromatography (CH_2Cl_2, $R_f=$ 0.6, TLC, developed in iodine chamber) to yield compound **16** as a yellow oil (1.68 g, 7.67 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=5.70$–5.82 (m, 1H), 4.89–5.01 (m, 2H), 3.95–4.06 (m, 1H), 3.79–3.90 (m, 1H), 2.65 (d, $J=9$ Hz, 6H), 2.05 (q, $J=7.0$ Hz, 2H), 1.38–1.76 (m, 6H), 1.26 ppm (t, $J=7.0$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta=138.3$, 114.7, 59.0, 36.1, 33.2, 29.9, 25.8, 24.5, 21.6, 16.3 ppm; ^{31}P NMR (81 MHz, $CDCl_3$): $\delta=36.90$ ppm.

***N*^{2}-(4-(6-((dimethylamino)(ethoxy)phosphoryl)hexyl)-2,6-dimethylphenyl)-*N*^{1}-mesityl-4,5-dihydro-1H-imidazol-3-ium chloride 17**: 9-BBN (0.46 g, 1.86 mmol, 0.6 equiv) was weighed in the glovebox and added to a stirred solution of ethyl *P*-(hex-5-en-1-yl)-*N,N*-dimethylphosphonamidate (0.68 g, 3.10 mmol, 1.1 equiv) in dry THF (60 mL). The reaction mixture was stirred for 20 h at room temperature and then concentrated under vacuum. A mixture of $[PdCl_2(dppf)]$ catalyst (0.16 g, 0.20 mmol, 7 mol%) and compound **5** (1.15 g, 2.82 mmol) was added followed by degassed DMF (150 mL). After this mixture was stirred for 5 min, $K_3PO_4{\cdot}H_2O$ (0.73 g, 3.44 mmol, 1.11 equiv) was added, and the reaction mixture was stirred for a further 10 min. Degassed H_2O (8 mL) was added, and the reaction mixture was stirred for 20 h at 100 °C (conversion was monitored by TLC: CH_2Cl_2/CH_3OH, 9:1). The reaction mixture was concentrated under vacuum at 65 °C, and the residue was azeotroped once with heptane and purified by column chromatography (CH_2Cl_2/CH_3OH, 9:1, $R_f=0.45$) to yield compound **17** as a yellow oil (0.55 g, 1.0 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.84$ (s, 1H), 6.93 (d, $^3J=5.5$ Hz, 4H), 4.64 (br s, 4H), 3.90–4.02 (m, 1H), 3.75–3.88 (m, 1H), 2.63 (d, $^3J=9.0$ Hz, 6H), 2.51 (t, $^3J=$ 7.5 Hz, 2H), 2.39 (d, $^3J=5.0$ Hz, 12H), 2.27 (s, 3H) 1.41–1.88 (m, 7H), 1.20–1.41 ppm (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta=159.1$, 145.4, 140.7, 135.0, 130.1, 129.4, 59.0, 53.4, 52.2, 36.1, 35.4, 30.8, 28.7, 25.9, 24.6, 22.0, 21.1, 18.2, 16.3 ppm; ^{31}P NMR (162 MHz, $CDCl_3$): $\delta=36.94$ ppm.

Metallation and synthesis of inhibitors

Complex 9: As a representative synthesis, in the glovebox, potassium hexamethyldisilazide (KHMDS, 0.5 M solution in toluene, 0.5 mL, 0.238 mmol, 1.1 equiv) was slowly added to a stirred suspension of preligand **7** (110 mg, 0.217 mmol, 1 equiv) in toluene (25 mL) at room temperature, and the mixture was left stirring for 16 h. A solution of Hoveyda–Grubbs I complex (117 mg, 0.195 mmol, 0.9 equiv) in toluene (5 mL) was added to the resultant orange solution. The brown reaction mixture was taken out of the glovebox and heated to 65 °C for 2 h, whilst stirring; it was then allowed to cool to room temperature and stirred for a further 48 h under inert conditions to give a dark green-brown colored solution. The volume was reduced to about 10 mL under vacuum, and the solution was filtered by cannula. All volatiles were evaporated, and the product was purified by column chromatography (degassed acetone) collecting the light green band to give complex **9** as a green oil (58 mg, 74 mmol, 38%). $R_f=0.7$ (degassed acetone); ^{1}H NMR (400 MHz, CD_2Cl_2): $\delta=16.51$, 16.45, (s, Ru=CH, 1H), 7.54, 7.08, 7.07, 6.97, 6.95, 6.93, 6.84, 6.82, (ArH, 8H), 4.83 (d, $J=4$ Hz, $CH(CH_3)_2$, 1H), 4.52 (m, CH_2CH_3, 2H), 4.15 (s, $N(CH_2)_2N$, 4H), 2.74 (m, $ArCH_2$, 2H), 2.69, 2.67, 2.12 (s, $ArCH_3$, 15H), 2.45, 2.43 (s, $N(CH_3)_2$, 6H), 2.41 (m, PCH_2, 2H), 1.46 (d, $J=4$ Hz, $CH(CH_3)_2$, 6H), 1.28 (m, $ArCH_2CH_2$, 2H), 1.22 ppm (m, CH_2CH_3, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta=$ 295.2, 210.7, 206.3, 154.8, 151.9, 145.0, 142.6, 138.8, 131.8, 129.5, 129.3, 129.2, 128.7, 122.3, 122.1, 113.0, 112.9, 75.1, 73.6, 70.6, 58.9, 51.4, 36.4, 36.3, 35.9, 30.5, 28.9, 25.6, 24.2, 20.8, 20.7, 19.1, 16.0 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): $\delta=35.83$ ppm; HRMS (ESI): *m/z* calcd for $[M-Cl^-]^+$: 754.2478; found: 754.2560.

Inhibitor 1: As a representative synthesis, a solution of hydrogen chloride in Et_2O (1 M, 0.65 mL, 0.65 mmol) was added by syringe to a stirred solution of complex **9** (50 mg, 0.066 mmol) in CH_2Cl_2 (5 mL) under Schlenk techniques, and the solution was stirred at room temperature for 3 h. The volatiles were removed under vacuum, CH_2Cl_2 (5 mL) was added, and a prepared solution of *p*-nitrophenol (9.2 mg, 0.066 mmol) and Et_3N (0.020 mL, 0.14 mmol) in CH_2Cl_2 (1 mL) was slowly added at room temperature, and the mixture was stirred for 2 h. The volatiles were removed under vacuum, and the product was purified by column chromatography (degassed acetone) collecting the front green band to give complex **1** as a bright green, dense oil (70%). ^{1}HNMR (400 MHz, CD_2Cl_2): $\delta=$ 16.54 (s, Ru=CH, 1H), 8.10 (d, $J=8$ Hz, $OArNO_2$, 2H), 6.94 (d, $J=$ 8 Hz, $OArNO_2$, 2H), 7.40, 7.38, 7.07, 6.90, 6.87, 6.82, 6.80 (m, ArH, 8H), 4.82, 4.81 (sep, $CH(CH_3)_2$, 1H), 4.55, 4.51 (m, OCH_2CH_3, 2H), 4.16 (bs, NCH_2CH_2N, 4H), 3.98 (m, $ArCH_2$, 2H), 2.49–2.40 (m, CH_2P, 2H), 2.13, 2.12, 1.47 (s, $ArCH_3$, 12H), 1.45 (d, $J=8$ Hz, $CH(CH_3)_2$, 6H), 1.33, 1.26, 1.23 ppm (m, $ArCH_2CH_2$, 2H); ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta=302.19$, 210.57, 206.68, 155.56, 154.93, 151.95, 144.56, 141.65, 138.91, 129.47, 129.26, 128.65, 125.96, 125.59, 122.28, 122.09, 121.04, 121.04, 121.00, 115.56, 112.91, 75.05, 73.62, 70.69, 63.20, 50.39, 30.57, 26.19, 24.79, 20.93, 19.13 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): $\delta=29.90$ ppm. HRMS (ESI): *m/z* calcd for $[M]^+$: 883.1858; found: 883.1739.

Complex 10: In accordance with the synthesis of complex **9**, **8** (78 mg, 0.129 mmol), KHMDS (0.26 mL, 0.5 M solution in toluene), Hoveyda–Grubbs I (59.6 mg, 0.099 mmol) were used, and column chromatography (degassed acetone then MeOH) gave complex **10** as a bright green, dense oil (45 mg, 51%). ^{1}H NMR (400 MHz, CD_2Cl_2): $\delta=16.41$, 16.36 (s, Ru=CH, 1H), 7.53–7.50, 7.38, 7.19, 7.04–6.86 (ArH, 9H), 4.90 (m, $OCH(CH_3)_2$, 1H), 4.16–3.98 (bs, NCH_2CH_2N, 4H), 3.56-3.44 (m, CH_2CH_3, 2H), 3.42–2.81 (m, $ArCH_2CH_2CH_2$, 6H), 2.68 (m, $OCH(CH_3)_2$, 6H), 2.12, 1.25 (s, $N(CH_3)_2$, 6H), 1.23, 1.22 (d, $J=4$ Hz, $ArCHCH_3$, 24H), 1.14 ppm (m, CH_2CH_3, 6H); ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta=289.30$, 213.76, 213.09, 163.45, 152.13, 149.14, 147.67, 147.66, 147.42, 144.03, 142.41, 142.30, 129.13, 124.19, 122.19, 121.95, 112.84, 74.93, 65.593, 58.927, 58.861, 48.892, 36.789, 36.631, 35.923, 35.883, 30.537, 28.664, 26.250, 23.897, 23.046, 21.455, 16.179, 16.114 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): $\delta=$ 37.97 ppm; HRMS (ESI): *m/z* calcd for $[M+H]^+$: 888.3341; found: 888.3325; *m/z* calcd for $[M-Cl^-]^+$: 852.3583; found: 852.3556.

Inhibitor 2: In accordance with the synthesis of inhibitor **1**, **10** (15.9 mg, 0.018 mmol), HCl (0.358 mmol, 0.358 mL), *p*-nitrophenol (2.5 mg, 0.018 mmol), and triethylamine (0.18 mmol, 0.025 mL) were used. Column chromatography (degassed acetone) gave inhibitor **2** as a green, dense oil (10 mg, 57%). ^{1}H NMR (400 MHz, CD_2Cl_2) $\delta=16.43$ (s, Ru=CH, 1H), 8.21 (d, $J=8$ Hz, $OArHNO_2$, 2H), 8.12 (d, $J=8$ Hz, $OArHNO_2$, 2H), 7.41-7.37, 7.17, 7.00–6.85, 6.81 (m,

ArH, 9H), 4.87 (m, OC*H*(CH_3)$_2$, 1H), 4.30–4.18 (m, CH_2CH$_3$, NCH_2CH_2N, 6H), 3.56 (m, ArC*H*(CH_3)$_2$, 4H) 3.44, 2.87 (m, ArCH_2, 2H), 2.62, 2.15, 2.13, 1.97 (m, ArCH_2CH_2, 2H), 1.26 (bs, ArCH(CH_3)$_2$, 24H) 1.22 (d, J=4 Hz, OCH(CH_3)$_2$, 6H), 1.14 ppm (m, CH_2CH_3, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ=285.22, 212.97, 162.08, 152.15, 149.40, 148.99, 144.01, 142.00, 129.56, 129.27, 125.96, 125.58, 124.33, 124.11, 122.17, 121.92, 121.05, 121.00, 120.92, 115.52, 112.88, 74.93, 63.24, 52.83, 29.63, 28.96, 28.80, 28.58, 26.51, 26.12, 23.15, 22.91, 21.46, 16.18, 16.12 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): δ=30.10 ppm; HRMS (ESI): *m/z* calcd for $[M-Cl^-]^+$: 946.3265; found: 946.3100.

Complex 18: In accordance with the synthesis of complex **9**, **17** (70 mg, 0.127 mmol) and KHMDS (0.25 mL, 0.5 M solution in toluene) were stirred for 5 h (this first step of the reaction requires less stirring time due to the better solubility of compound **17** in toluene, in comparison with compounds **7** and **8**), Hoveyda–Grubbs I (72.0 mg, 0.12 mmol) was added, and column chromatography (degassed acetone) gave the complex **18** as a bright green oil (39 mg, 39%). ^{1}H NMR (400 MHz, CD_2Cl_2): δ=16.52, 16.46 (Ru=CH, 1H), 7.54, 7.07, 6.97, 6.95, 6.90, 6.82 (ArH, 8H), 4.88 (m, CH_2CH_3, 2H), 4.15 (s, NCH_2CH_2N, 4H), 3.95, 3.85 (m, C*H*(CH_3)$_2$, 1H), 2.66, 2.64 (s, ArCH_3, 15H), 2.60 (m, ArCH_2, 2H), 2.44, 2.40 (s, N(CH_3)$_2$, 6H), 1.84–1.64 (tether –CH_2–, 10H) 1.46 (d, J=8 Hz, CH(CH_3)$_2$, 6H), 1.21 ppm (m, CH_2CH_3, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ=295.15, 211.06, 210.63, 152.02, 151.92, 145.22, 145.06, 143.79, 138.80, 129.52, 129.31, 128.57, 122.46, 122.22, 122.07, 113.00, 112.87, 75.04, 58.76, 52.84, 35.84, 35.53, 30.58, 29.03, 26.27, 25.82, 24.53, 22.17, 20.89, 16.08 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): δ=36.37 ppm.

Inhibitor 3: In accordance with the synthesis of inhibitor **1**, complex **18** (11.8 mg, 0.0142 mmol), HCl (0.14 mmol, 0.142 mL), *p*-nitrophenol (2 mg, 0.0142 mmol), and triethylamine (0.18 mmol, 0.025 mL) gave complex **3** as a green oil (97%). ^{1}H NMR (400 MHz, CD_2Cl_2): δ=16.55, 16.47 (Ru=CH, 1H), 8.20, 8.09, 7.51, 7.37, 7.06, 6.93, 6.87, 6.82 (ArH, 12H), 4.87 (m, CH_2CH$_3$, 2H), 4.20 (m, C*H*(CH_3)$_2$, 1H), 4.16 (s, NCH_2CH_2, 4H), 2.65 (m, ArCH_2, 2H), 2.44, 2.40 (s, ArCH_3, 15H), 2.01, 1.70, 1.57, 1.49, 1.33 (m, tether –CH_2–, 10H), 1.21 ppm (d, CH_2CH_3, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ=298.14, 210.96, 207.58, 161.64, 152.23, 145.23, 143.46, 141.46, 138.95, 129.66, 129.35, 128.68, 126.14, 125.72, 122.61, 122.20, 121.00, 120.95, 120.40, 115.84, 112.92, 75.00, 63.23, 51.60, 35.50, 30.94, 30.27, 28.86, 26.64, 25.25, 22.19, 21.11, 21.08, 16.34 ppm; ^{31}P NMR (162 MHz, CD_2Cl_2): δ=30.39 ppm; HRMS (ESI): *m/z* calcd for $[M]^+$: 925.2327; found: 925.2250.

Hybridization reactions

In a typical procedure, a solution of inhibitor **1**, **2**, or **3** (10 equiv with respect to cutinase) in degassed CH_2Cl_2 (0.5 mL) was slowly added by syringe to a stirred solution of cutinase (50 μM in degassed Tris-HCl pH 8.5 or 50 mM in sodium acetate buffer pH 5); the volume of CH_2Cl_2 was 1/19 of the volume of buffer so that it accounted for 5% v/v in the mixture. The reaction was stirred vigorously at 25 °C for 24 h. The mixture was transferred to a 250 mL round bottom flask and placed on a rotary evaporator with a fast spin at 25 °C to remove CH_2Cl_2. The solids were filtered off three times, and the filtrate was transferred to Vivaspin 6 centrifugation tubes with PEG membrane of 10 kDa MWCO and centrifuged (3× 1.5 h at 3500 rpm) for dialysis, the volume was restored each time with Milli-Q water and finally with buffer (50 mM) to 9.5 mL. The resulting solutions were stored at −20 °C in degassed vessels. For ESI-MS analyses, an aliquot of 0.5 mL was further dialyzed and treated with formic acid for its denaturation before injection.

Catalytic reactions with metalloprotein hybrids

In a typical experiment, *N,N*-diallyl-*p*-toluenesulfonamide (TDA) or allylbenzene and bibenzyl (substrates and internal standard, respectively, were mixed in a stock solution of 10 mM of each component in degassed CH_2Cl_2) were added to a Schlenk flask (1 mL, 1 μmol, 1 equiv) that was charged with a stirring bar and $MgCl_2$ (1 mg, 10.5 μmol, 10.5 equiv; optionally). In the reactions with allylbenzene, tetrabutylammonium chloride (TBACl, 2.5 mg, 9 μmol) was added too. The solvent was removed under vacuum, and the flask was refilled with N_2 gas. The catalytic hybrid (solution of 50 μM in buffer) was added to the Schlenk flask (1 mL, 0.05 μmol, 0.05 equiv) followed by the slow addition of degassed CH_2Cl_2 (0.05 mL) by syringe while stirring (final total volume = 1.05 mL, which made CH_2Cl_2 5% *v/v* and TBACl ≈5% *w/w*; final concentrations: TDA or allylbenzene = 0.95 mM, bibenzyl = 0.95 mM, catalytic hybrid = 47.6 μM, buffer = 47.6 mM, $MgCl_2$ = 10 mM). The solution was stirred vigorously for 20 h at room temperature. The organic phase was extracted with CH_2Cl_2 (3×6 mL), concentrated under vacuum, and dissolved in CH_2Cl_2 (0.05 mL) for GC analysis.

Acknowledgements

The authors thank the Dutch National Research School Combination-Catalysis (NRSCC) for the financial support.

Keywords: enzyme catalysis · lipase · metalloenzymes · metathesis · ruthenium

[1] a) F. Yu, V. M. Cangelosi, M. L. Zastrow, M. Tegoni, J. S. Plegaria, A. G. Tebo, C. S. Mocny, L. Ruckthong, H. Qayyum, V. L. Pecoraro, *Chem. Rev.* **2014**, *114*, 3495–3578; b) J. C. Lewis, *ACS Catal.* **2013**, *3*, 2954–2975; c) D. Qi, C.-M. Tann, D. Haring, M. D. Distefano, *Chem. Rev.* **2001**, *101*, 3081–3111; d) M. Creus, T. R. Ward, *Org. Biomol. Chem.* **2007**, *5*, 1835–1844; e) J. Steinreiber, T. R. Ward, *Coord. Chem. Rev.* **2008**, *252*, 751–766; f) P. J. Deuss, R. den Heeten, W. Laan, P. C. J. Kamer, *Chem. Eur. J.* **2011**, *17*, 4680–4698.

[2] a) T. R. Ward, *Bio-inspired Catalysis*, **2009**, Springer-Verlag, Berlin, Heidelberg; b) M. Skander, N. Humbert, J. Collot, J. Gradinaru, G. Klein, A. Loosli, J. Sauser, A. Zocchi, F. Gilardoni, T. R. Ward, *J. Am. Chem. Soc.* **2004**, *126*, 14411–14418.

[3] a) H. L. Levine, E. T. Kaiser, *J. Am. Chem. Soc.* **1978**, *100*, 7670–7677; b) H. L. Levine, Y. Nakagawa, E. T. Kaiser, *Biochem. Biophys. Res. Commun.* **1977**, *76*, 64–70; c) E. T. Kaiser, D. S. Lawrence, *Science* **1984**, *226*, 505–511; d) R. R. Davies, M. D. Distefano, *J. Am. Chem. Soc.* **1997**, *119*, 11643–11652; e) R. R. Davies, H. Kuang, D. Qi, A. Mazhary, E. Mayaan, M. D. Distefano, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 79–84; f) J. R. Carey, S. K. Ma, T. D. Pfister, D. K. Garner, H. K. Kim, J. A. Abramite, Z. Wang, Z. Guo, Y. Lu, *J. Am. Chem. Soc.* **2004**, *126*, 10812–10813; g) J.-L. Zhang, D. K. Garner, L. Liang, Q. Chen, Y. Lu, *Chem. Commun.* **2008**, 1665–1667.

[4] a) M. J. Boerakker, J. M. Hannink, P. H. Bomans, P. M. Frederik, R. J. M. Nolte, E. M. Meijer, N. A. J. M. Sommerdijk, *Angew. Chem. Int. Ed.* **2002**, *41*, 4239–4241; *Angew. Chem.* **2002**, *114*, 4413–4415; b) A. Mahammed, Z. Gross, *J. Am. Chem. Soc.* **2005**, *127*, 2883–2887; c) M. Ohashi, T. Koshiyama, T. Ueno, M. Yanase, H. Fujii, Y. Watanabe, *Angew. Chem. Int. Ed.* **2003**, *42*, 1005–1008; *Angew. Chem.* **2003**, *115*, 1035–1038.

[5] S. Kanbak-Aksu, M. Nahid Hasan, W. R. Hagen, F. Hollmann, D. Sordi, R. A. Sheldon, I. W. C. E. Arends, *Chem. Commun.* **2012**, *48*, 5745–5747.

[6] I. Drienovská, A. Rioz-Martínez, A. Draksharapua, G. Roelfes, *Chem. Sci.* **2015**, *6*, 770–776.

[7] a) C. A. Kruithof, M. A. Casado, G. Guillena, M. R. Egmond, A. van der Kerk-van Hoof, A. J. R. Heck, R. J. M. Klein Gebbink, G. van Koten, *Chem. Eur. J.* **2005**, *11*, 6869; b) B. Wieczorek, A. Träff, P. Krumlinde, H. P. Dijkstra, M. R. Egmond, G. van Koten, J.-E. Bäckvall, R. J. M. Klein Gebbink, *Tetrahedron Lett.* **2011**, *52*, 1601; c) M. T. Reetz, M. Rentzsch, A. Pletsch, M. Maywald, *Chimia* **2002**, *56*, 721–723.

[8] T. Matsuo, C. Imai, T. Yoshida, T. Saito, T. Hayashi, S. Hirota, *Chem. Commun.* **2012**, *48*, 1662 – 1664.
[9] a) H. P. Dijkstra, H. Sprong, B. N. H. Aerts, C. A. Kruithof, M. R. Egmond, R. J. M. Klein Gebbink, *Org. Biomol. Chem.* **2008**, *6*, 523 – 531; b) B. Wieczorek, B. Lemcke, H. P. Dijkstra, M. R. Egmond, R. J. M. Klein Gebbink, G. van Koten, *Eur. J. Inorg. Chem.* **2010**, 1929 – 1938.
[10] L. Rutten, B. Wieczorek, J.-P. B. A. Mannie, C. A. Kruithof, H. P. Dijkstra, M. R. Egmond, M. Lutz, R. J. M. Klein Gebbink, P. Gros, G. van Koten, *Chem. Eur. J.* **2009**, *15*, 4270 – 4280.
[11] M. Basauri-Molina, C. F. Riemersma, M. A. Würdemann, H. Kleijn, R. J. M. Klein Gebbink, *Chem. Commun.* **2015**, *51*, 6792 – 6795.
[12] J. Pierron, C. Malan, M. Creus, J. Gradinaru, I. Hafner, A. Ivanova, A. Sardo, T. R. Ward, *Angew. Chem. Int. Ed.* **2008**, *47*, 701 – 705; *Angew. Chem.* **2008**, *120*, 713 – 717.
[13] J. Bos, F. Fusetti, A. J. M. Driessen, G. Roelfes, *Angew. Chem. Int. Ed.* **2012**, *51*, 7472 – 7475; *Angew. Chem.* **2012**, *124*, 7590 – 7593.
[14] a) Y. A. Lin, J. M. Chalker, N. Floyd, G. J. L. Bernardes, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 9642 – 9643; b) K. Kirshenbaum, P. S. Arora, *Nat. Chem. Biol.* **2008**, *4*, 527 – 528; c) Y. A. Lin, J. M. Chalker, B. G. Davis, *J. Am. Chem. Soc.* **2010**, *132*, 16805 – 16811; d) C. A. Denard, H. Huang, M. J. Bartlett, L. Lu, Y. Tan, H. Zhao, J. F. Hartwig, *Angew. Chem. Int. Ed.* **2014**, *53*, 465 – 469; *Angew. Chem.* **2014**, *126*, 475 – 479.
[15] A. J. Jiang, Y. Zhao, R. R. Schrock, A. H. Hoveyda, *J. Am. Chem. Soc.* **2009**, *131*, 16630 – 16631.
[16] C. Mayer, D. G. Gillingham, T. R. Ward, D. Hilvert, *Chem. Commun.* **2011**, *47*, 12068 – 12070.
[17] a) F. Philippart, M. Arlt, S. Gotzen, S.-J. Tenne, M. Bocola, H.-H. Chen, L. Zhu, U. Schwaneberg, J. Okuda, *Chem. Eur. J.* **2013**, *19*, 13865 – 13871; b) D. F. Sauer, M. Bocola, C. Broglia, M. Arlt, L.-L. Zhu, M. Brocker, U. Schwaneberg, J. Okuda, *Chem. Asian J.* **2015**, *10*, 177 – 182.
[18] a) C. Lo, M. R. Ringenberg, D. Gnandt, Y. Wilson, T. R. Ward, *Chem. Commun.* **2011**, *47*, 12065 – 12067; b) A. Kajetanowicz, A. Chatterjee, R. Reuter, T. R. Ward, *Catal. Lett.* **2014**, *144*, 373 – 379.
[19] a) J. P. Jordan, R. H. Grubbs, *Angew. Chem. Int. Ed.* **2007**, *46*, 5152 – 5155; *Angew. Chem.* **2007**, *119*, 5244 – 5247; b) J. B. Binder, J. J. Blank, R. T. Raines, *Org. Lett.* **2007**, *9*, 4885 – 4888.
[20] L. M. Mannesse, R. C. Cox, B. C. Koops, H. M. Verheij, G. H. de Haas, M. R. Egmond, H. T. W. M. van der Hijden, J. de Vlieg, *Biochemistry* **1995**, *34*, 6400 – 6407.
[21] The van der Waals volumes of the residues $-CH_3$ and $-CH(CH_3)_2$ are 5.873 and 8.988 $Å^3$, respectively, and the furthest length difference between hydrogens in mesityl versus 2,6-diisopropylbenzene is 6.23 Å (models built with YASARA, see ref. [26]).
[22] T. Matsuo, T. Yoshida, A. Fujii, K. Kawahara, S. Hirota, *Organometallics* **2013**, *32*, 5313 – 5319.
[23] S. J. P'Poo, H.-J. Schanz, *J. Am. Chem. Soc.* **2007**, *129*, 14200 – 14212.
[24] S. B. Petersen, P. Fojan, E. I. Petersen, M. T. Neves Petersen, *J. Biomed. Biotechnol.* **2001**, *1*, 62 – 69.
[25] C. Martinez, P. de Geus, M. Lauwereys, G. Matthyssens, C. Cambillau, *Nature* **1992**, *356*, 615 – 618.
[26] Calculated using YASARA, see E. Krieger, G. Koraimann, G. Vriend, *Proteins Struct. Funct. Bioinf.* **2002**, *47*, 393 – 402.
[27] M. R. Egmond, J. de Vlieg, *Biochimie* **2000**, *82*, 1015 – 1021.
[28] A. L.-F. Chow, M. H. So, W. Lu, N.-Y. Zhu, C.-M. Che, *Chem. Asian J.* **2011**, *6*, 544 – 553.

Received: June 19, 2015
Published online on September 8, 2015