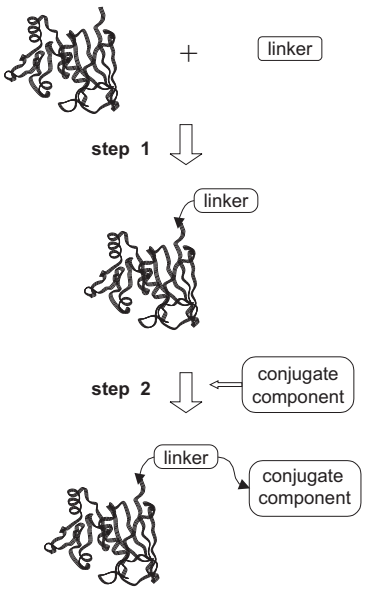
Bioconjugation of Peptides by Palladium-Catalyzed C–C Cross-Coupling in Water**

Harald Dibowski and Franz P. Schmidtchen*

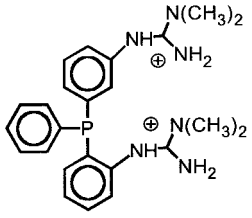
The biological activity of proteins in higher organisms frequently depends on their covalent modification with other substructures (e.g. prosthetic groups, saccharide chains, and membrane anchors). The attachment is performed in vivo at the enzymatic level and therefore cannot be directly controlled by methods of gene technology. An in vitro method for regioselectively attaching non-proteinogenic structural elements to a native folded protein would be a highly desirable tool supplementing the use of suitable cell lines. An obvious strategy would encompass a two-step process in which an amino acid containing some unique functionality is incorporated first into the primary sequence at the desired position (in Scheme 1 this is the N terminus of a folded protein) in a known manner.[1] In the second step this substructure would have to be linked with the second component to give the final bioconjugate (Scheme 1).



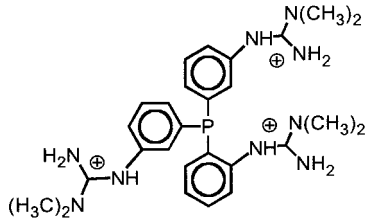
Scheme 1. Formation of a bioconjugate via an intermediate.

This second non-enzymatic step, apart from being chemoselective and establishing a permanent linkage of the two substructures, should allow coupling under mild reaction conditions in water to ensure that the fragile tertiary structure of the proteins is not damaged. Moreover, a high reactivity (rate constant) is mandatory in order to allow the bimolecular coupling to take place at the very small molar concentrations of the biopolymers within reasonable periods of time. Here we report that the palladium-catalyzed cross-coupling of aryl iodides with terminal alkynes, the Castro–Stephens–Sonogashira reaction[5] using a palladium–guanidinophosphane catalyst formed in situ, complies with these demands.

Water-soluble palladium catalysts for C–C cross-coupling usually employ sulfonated anionic triphenylphosphanes as ligands.[6] Under the basic reaction conditions required for Sonogashira reactions the proteins themselves carry a net negative charge as well, so that the catalytic process would have to overcome an electrostatic barrier. Experimental data indeed show that the catalytic activity of the cationic guanidinophosphanes **1** and **2**, which we recently synthesized, is well enhanced. In addition, these novel ligands are just as hydrophilic and substantially more stable against oxidation than their sulfonated analogues.[7]

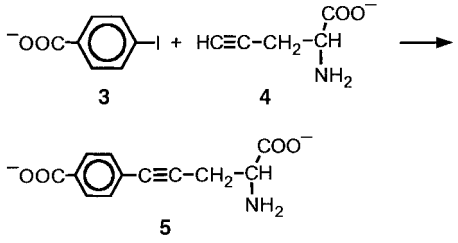


1



2

In preliminary experiments nonnatural free amino acids containing alkyne or iodoaryl groups were allowed to react with the corresponding reaction partners in aqueous acetonitrile (H_2O:CH_3CN = 7:3) at 50 °C. The reaction of *p*-iodobenzoate (**3**) with propargylglycine (**4**) gave the cross-coupled product **5** in 75 % yield after about three hours [Reaction (1)]



3 **4**

5

(1)

without formation of the product from alkyne homo-coupling. Under the same conditions (see Table 1) 3-iodotyrosine (**6**) reacted with propiolic acid [**7**; Reaction (2)] in 25 hours to give the C–C coupled product **8** (86 % yield), which could be isolated after acidic work-up and characterized by NMR spectroscopy. Longer reaction times or basic work-up caused the primary product **8** to rearrange cleanly in a known fashion to the benzofuran derivative **9**.

The same reaction course was observed under purely aqueous conditions (3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid (TAPS) buffer, pH 8.3); therefore, the stage was set to test if the reaction conditions employed were influenced by the protein or were compatible with its catalytic function. We chose RNase A as our model protein, since this nuclease is readily available and its activity can be assayed

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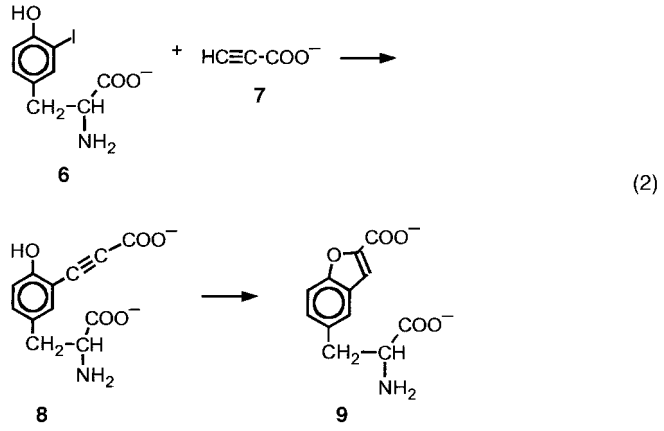
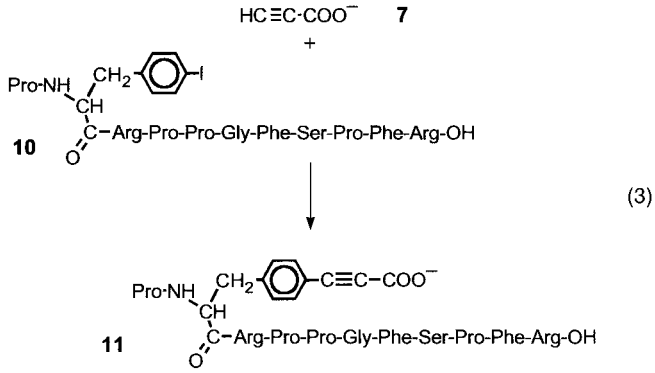


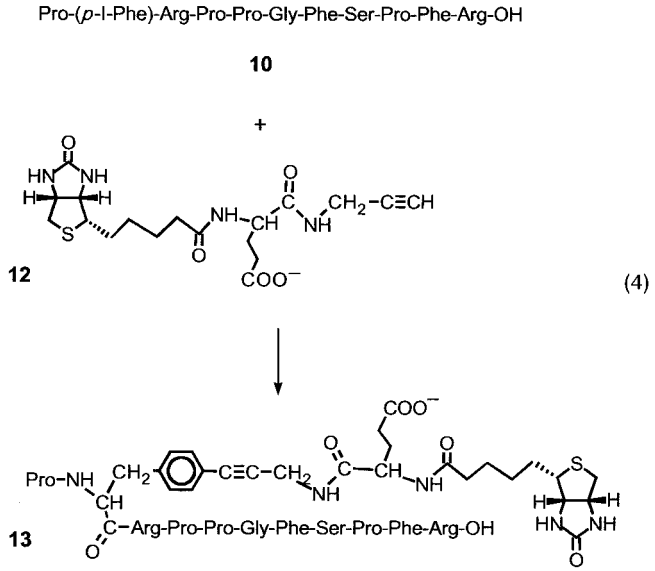
Table 1. Reaction conditions for the cross-couplings.[a]

Reaction	Solvent	Catalyst [mol%]	Yield [%]	*t* [min]
(1)	H_2O:CH_3CN = 7:3 $N(C_2H_5)_3$[b]	5	75	195
(2)	H_2O:CH_3CN = 7:3 $N(C_2H_5)_3$	5	86	120
(2)	0.2 M TAPS buffer pH 8.3 in H_2O	5[c]	89	140
(3)	0.2 M TAPS buffer pH 8.3 in H_2O	10[d]	75	240
(4)	0.2 M TAPS buffer pH 8.3 in H_2O	10	75	240

[a] T = 50 °C, unless noted otherwise. Substrate concentrations: iodoarene 10 mM, alkyne 20 mM. The catalyst solution was generated in situ by first mixing a solution containing 10 mM $Pd(OAc)_2$ and 50 mM **1**·Cl^- in water with 0.1 M CuI in acetonitrile in a volumetric ratio of 5:1 and then conditioning at 4° C under N_2 for five days. The activity of this stock solution was checked in regular intervals in standard catalysis reactions and remained constant for several weeks. [b] Iodoarene:alkyne = 1:1.4 with addition of four equivalents of hydroxylamine. [c] Ligand **2**. [d] Only four equivalents of ligand and one equivalent of CuI in the catalyst solution, T = 35 °C.



In close analogy peptides can be conjugated with biotin derivatives to provide a new access to the biotin–avidin technology[9] widely used in biological chemistry. In a demonstration biotinylglutamoylpropargylamide (**12**), a water-soluble biotin derivative, was allowed to react with **10** in aqueous TAPS buffer [pH 8.3; Reaction (4)]. After four



quickly by standard tests.[8] A comparison of the kinetics of Reaction (2) with and without the addition of RNase A (14 mg mL^{-1}) gave no qualitative difference in product formation, but the reaction in the presence of protein was markedly faster. The nuclease activity as well as its capillary electrophoretic behavior was determined before and after the reaction; it was not influenced at all by the palladium-catalyzed cross-coupling.

As an example of the regioselective C–C coupling of a multifunctional, free and unprotected peptide, Pro(*p*-I-Phe)-bradykinin (**10**) was allowed to react with propiolic acid [**7**; Reaction (3)]. The synthetic undecapeptide **10** itself can be synthesized in good yield from Pro(*p*-I-Phe)NH_2 and bradykinin[4a] by kinetically controlled reverse proteolysis with cathepsin C. Monitoring the reaction in aqueous TAPS buffer (pH 8.3) at 35 °C showed the clean formation of a single product in 91% yield after three hours. After it was isolated by HPLC, this compound was identified by FAB-MS (m/z = 1373; M^+) as the desired cross-coupling product.

hours a single product was formed in 75% yield, which was purified by gel chromatography and showed correct signals for the desired cross-coupling product in the electrospray mass spectrum [m/z = 1713 (M+H$^+$), 857 (M+2H$^+$), 572 (M+3H$^+$)]. The proof that this product possesses a competent biotin substructure was accomplished by affinity capillary electrophoresis (Figure 1). With incremental addition of the biotin-specific binding protein avidin to a sample of the reaction mixture that had not yet completely reacted, only the product peak was diminished to give a new protein complex.

The C–C cross-coupling catalyzed by palladium–guanidinoaryl–phosphane complexes thus allows the regioselective linking of iodoaryl and alkyne structures which are chemically rather stable and are orthogonal in their reactivity to almost all other functional groups in proteins. Their specific protection as well as general precautions for preserving the tertiary structure are therefore not necessary, since the native structure and function of the proteins remain unharmed under the extremely mild reaction conditions. A wide usage of

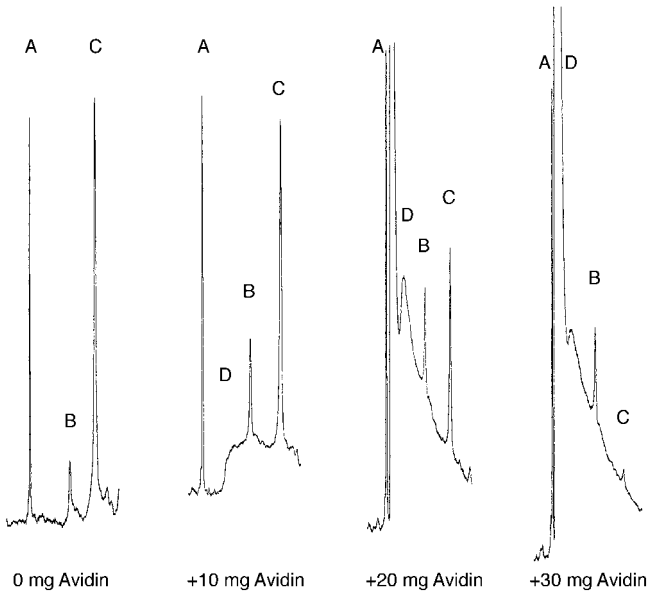


Figure 1. Affinity capillary electrophoresis of product **13** (C) from Reaction (4). A = catalyst complex, B = Pro(*p*-I-Phe)bradykinin (**10**), D = avidin – product complex. See text for further details.

this new method for the construction of structurally well-defined protein conjugates with nonnatural functions can be easily foreseen.

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[1] In principal, known procedures include, among others, total synthesis, peptide-segment condensation,[2] in vitro protein biosynthesis,[3] and reverse proteolysis.[4]

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A Self-Replicating Peptide under Ionic Control**

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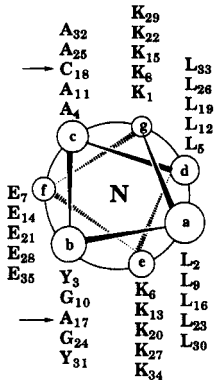
The self-replicating abilities of DNA, in addition to providing a genetic transfer mechanism for reproducing species, have played a significant role in the widespread development of biotechnology strategies.[1] DNA is unique in its self-replication, and species that rely on DNA for replication can adapt to environmental changes through natural selection. Recent examples of designed molecular systems capable of self-replication include nucleotide-based oligomers,[2] conjugates of adenine and Kemp's triacid,[3] peptides,[4] and micelles.[5] The production of a self-replicating molecule from a large molecular pool has been a more elusive target.[6] Recent work of Lee et al. demonstrated that peptides from the GCN4 leucine zipper domain self-replicate in an autocatalytic cycle.[4] We sought a peptidic self-replicating system that would be sensitive to environmental conditions and reproduce only under extreme conditions. We now describe a peptide that reproduces autocatalytically in an environmentally dependent manner.

The peptide K1K2 (Figure 1) was designed based on the sequence of the KK peptide of Zhou et al.[7] and on our peptide E1E2.[8] The K1K2 peptide contains Lys residues at

a)



b)

K1K2: Ac-KLYALKE-KLGALKE-KLACLKE-KLGALKE-KLYALKE-$CONH_2$

K1: Ac-KLYALKE-KLGALKE-KLA-COSR

K2: H_2N-CLKE-KLGALKE-KLYALKE-$CONH_2$

Figure 1. a) Helical wheel diagram of K1K2 showing the positions of the coiled-coil, heptad repeat (a – g). b) Peptide sequences employed in the study [R = $(CH_2)_2CONH_2$]. Ligation residues Ala and Cys are located at the solvent-exposed b and c positions, as indicated by the arrows.

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