Palladium-mediated *S*-arylation and *S*-allylation for late-stage peptide functionalization and protein conjugation

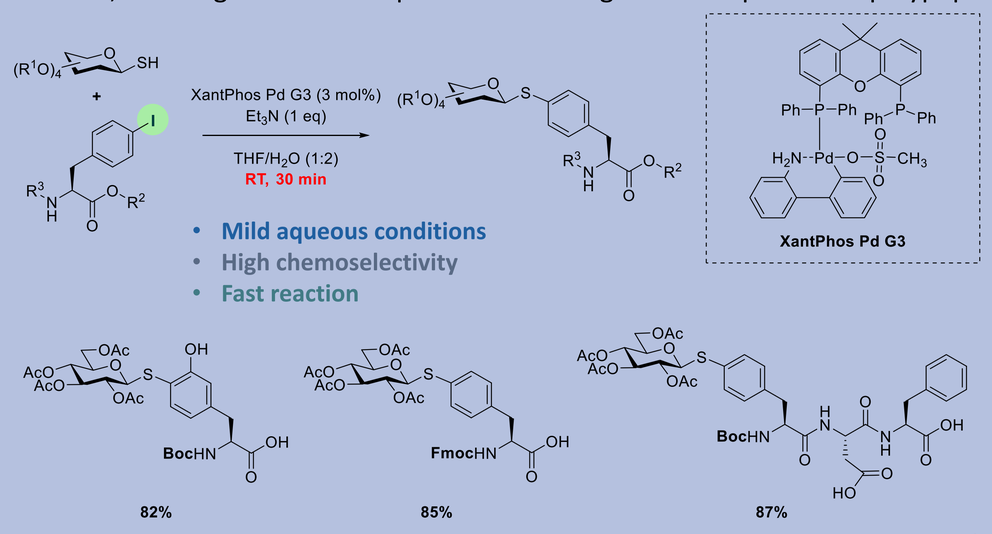
https://doi.org/10.17952/37EPS.2024.P2010

Ross D. Ballantine[a], Mingxiang Zhu[b], Ameni Hadj Mohamed[b], Aromal Asokan[a], Linhuan Shen[b], Mélanie Chenon[a], Ana Novak[a], Vincent Robert[c], Séverine Morisset-Lopez[a], Massimiliano Beltramo[c], Carlo Pifferi[a], Vincent Aucagne[a] & Samir Messaoudi[b].

[a] CBM, CNRS UPR4301, Rue Charles Sadron – 45071 Orléans, France, [b] BioCIS, CNRS UMR8076, 19 Avenue des Sciences – 91400 Orsay, France, [c] PRC, INRA UMR85 – 37380 Nouzilly, France

Chemoselective Functionalization *via* Buchwald-Hartwig-Migita

The chemoselective functionalization of peptides and proteins through manipulation and modification of the integral within a complex polyfunctional system, represents a key area of research for the elucidation of biological mechanisms and generation of potential pharmaceuticals. Messaoudi *et al*. recently reported the palladium catalysed Buchwald-Hartwig-Migita (BHM) coupling of thiosugars as well as aryl- and alkyl-thiols with various iodoarenes at room temperature under remarkably mild conditions. The coupling involves the use of the XantPhos Pd G3 as a precatalyst and exhibits a high functional group tolerance.[1] The synthetic utility of this method was subsequently expanded by using semi-aqueous conditions to perform various conjugation reactions on unprotected para-iodophenylalanine (pIPhe) containing peptides, with various glycosyl thiols.[2] The reactions were fast and highly chemoselective, allowing the use of unprotected thiosugars and unprotected polypeptides.



Scheme 1 – General conditions for synthesis of thioglyco-amino acids by Messaoudi *et al.*[1]

S-Arylation of pIPhe containing unprotected peptides

While *S*-arylation *via* BHM had previously been demonstrated for the synthesis of thioglycopeptides,[2] herein we extend the scope of this method to include the thiomethylation of a biologically relevant model peptide. The methodology is unprecedented in terms of mild reaction conditions which allow controlled late-stage peptide diversification. Importantly, an unreactive tag (iodo-aryl moiety) can be incorporated into peptide synthesis or protein expression[3] until the addition of palladium catalyst, conferring excellent bioconjugative control. Kisspeptin-10 (KP-10) (**1**) is a potent neural decapeptide that binds to G-protein-coupled receptor 54 (GPR54) in order to stimulate the release of gonadotropin-releasing hormone (GnRH). KP-10 (**1**) contains two aromatic residues which are crucial for target binding, therefore we sought to synthesise analogues whereby these residues were substituted for thiomethylated phenylalanine to potentially modulate activity for improved binding, higher selectivity towards related receptors or antagonistic activity.

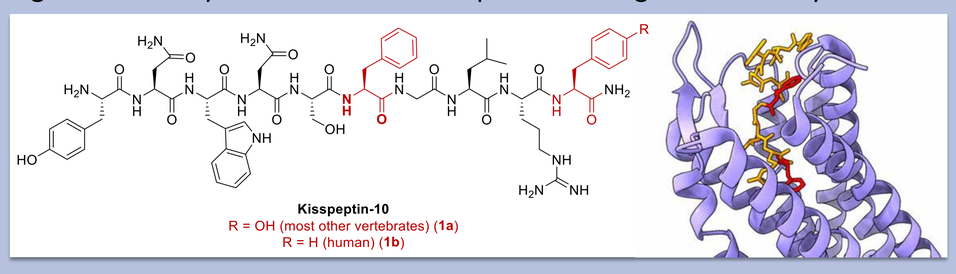
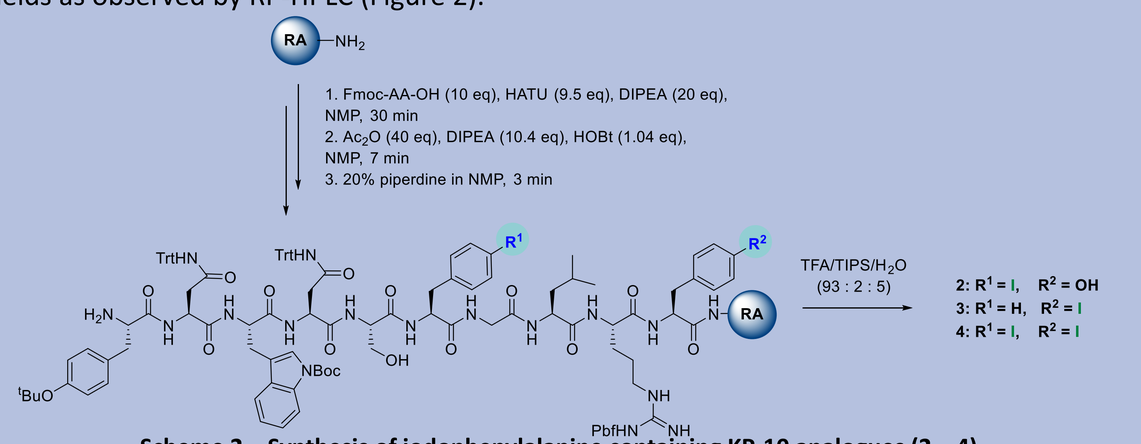
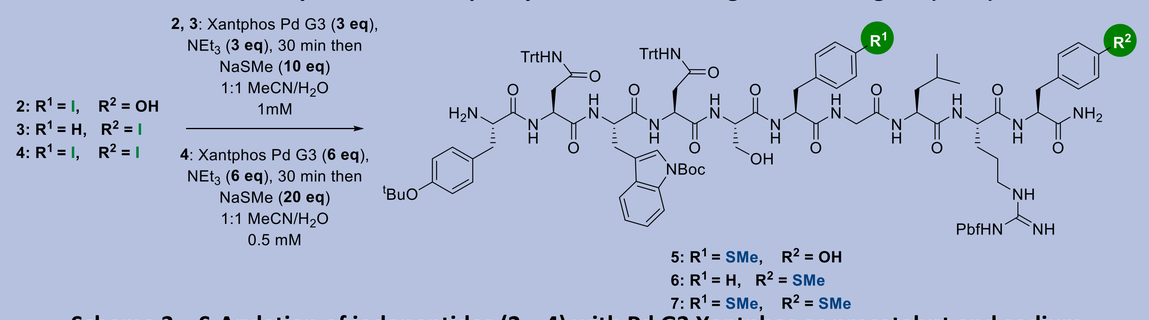


Figure 1 – Left: Structure of kisspeptin-10 (1) with residues important for binding in red, Right: Cryo-EM structure of kisspeptin receptor (purple) bound to KP-10 (1) PDB: 8ZJD[4]

Three analogues (**2** – **4**) were synthesized via automated Fmoc solid-phase peptide synthesis (SPPS) on Rink amide (RA) ChemMatrix resin (0.36 mmol/g), incorporating pIPhe alternately at position 6 (**2**) and 10 (**3**) and a double substituted analogue (**4**). The crude mono-iodopeptides were then reacted with Xantphos Pd G3 (3 eq) and trimethylamine (3 eq) for 30 minutes before addition of sodium thiomethoxide (10 eq). The reagents were doubled for crude di-iodopeptide (**4**). After 15 minutes, the reactions had proceeded to near quantitative yields as observed by RP-HPLC (Figure 2).

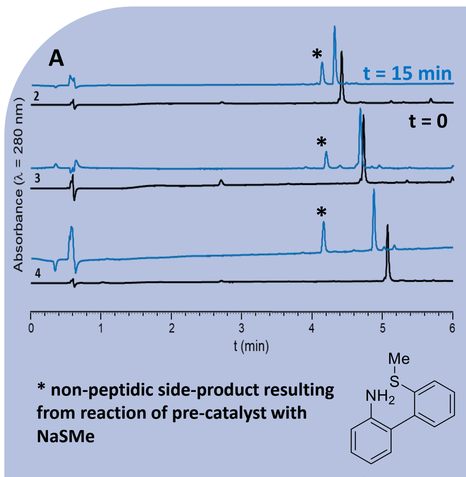


Scheme 2 – Synthesis of iodophenylalanine containing KP-10 analogues (2 – 4)



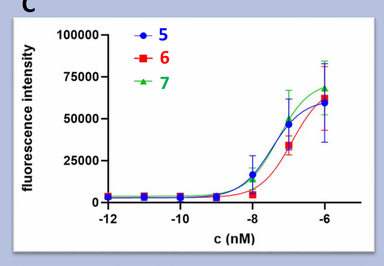
Scheme 3 – S-Arylation of iodopeptides (2 – 4) with Pd G3 Xantphos as precatalyst and sodium thiomethoxide as thiolate source

Peptides (**5** – **7**) were purified by semi-preparative RP-HPLC and obtained with an overall yield of 9 – 12%. Since KP10 has significant hydrophobic character and the reaction profiles are clean (> 99% conversion), it is likely that the loss of yield is due to aggregation of the peptides prior to purification. The yield was improved three-fold for KP10 6,10-SMe (**7**) by using 20% DMSO in the work-up, in order to disrupt any hydrophobic aggregation.



B

Analogue	Conversion %	Isolated Yield %	Isolated Yield % (20% DMSO workup)
KP10 6-SMePhe (5)	> 99	12	N/A
KP10 10-SMePhe (6)	> 99	9	N/A
KP10 6,10-SMePhe (7)	> 99	12	36



D

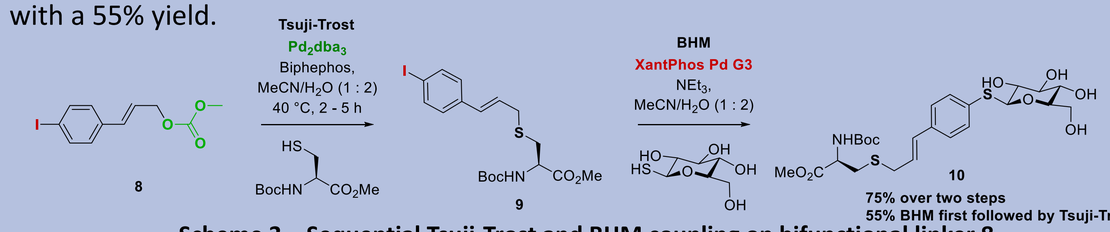
Analogue	EC50 / nM
KP10 (1)	2.5[5]
KP10 6-SMePhe (5)	50
KP10 10-SMePhe (6)	122
KP10 6,10-SMePhe (7)	33

Figure 2 – A: RP-HPLC traces of iodopeptides (2 – 4) in black, reaction profile after 15 minutes in blue (5-50% MeCN in H_2O with 0.1% TFA over 5 min) B: Percentage yields of thioaryl peptides (5 - 7) after purification, C: Fluorescence-based Ca^{2+} mobilization assay of compounds 5 – 7 against cells expressing the GPCR and D: Determined EC50 of analogues 5 – 7 compared with native KP10 (1)

The activity of compounds **5** – **7** was assessed *via* a fluorescence-based Ca^{2+} assay, whereby activation of the GPCR increases intracellular levels of calcium which are then detected by a calcium sensitive fluorophore resulting in fluorescence. Preliminary results show the analogues maintain nanomolar binding to GPR54, albeit slightly reduced compared to natural KP10 (**1**).

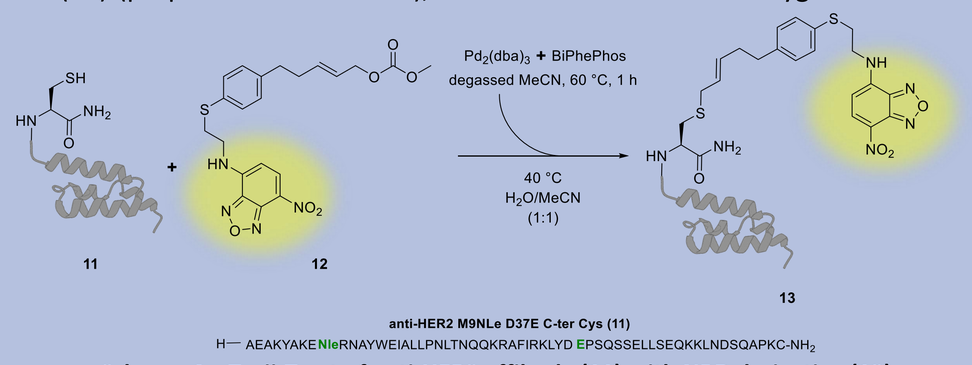
S-Arylation vs *S*-allylation

In addition to the BHM coupling, herein we demonstrate a Tsuji-Trost reaction mediated by tris(dibenzylideneacetone) dipalladium (0) ($Pd_2[dba]_3$) in the presence of a thiol source and an allyl carbamate. The two reactions are orthogonal to each other with chemoselectivity being controlled by the palladium catalyst, presenting a potential novel method for late stage peptide functionalization and protein conjugation. Preliminary results using bifunctional linker **8** under Tsuji-Trost conditions followed by BHM coupling afforded the desired product (**10**) in 75% yield over 2 steps. Inversely, initial BHM coupling followed by Tsuji-Trost resulted in **10** with a 55% yield.



Scheme 3 – Sequential Tsuji-Trost and BHM coupling on bifunctional linker 8

The methodology has since been applied to labelling an optimized anti-HER2 affibody (**11**) which was synthesized via automated Fmoc SPPS, installing a cysteine at the *C*-terminus.[6] A catalyst system was prepared by mixing Biphephos with $Pd_2(dba)_3$ in deoxygenated acetonitrile and heated to 60 °C for 1 hour. The catalyst solution was added to a solution containing affibody **11** and 7-nitrobenz-2-oxa-1,3-diazole (NBD) functionalized with an allyl carbamate (**12**) (prepared *via* BHM of **8**), then the reaction was deoxygenated and stirred at 40 °C.



anti-HER2 M9NLe D37E C-ter Cys (11)

H— AEAKYAKE**Nle**RNAYWEIALLPNLTNQQKRAFIRKLYD EPSQSSELLSEQKKLNDSQAPKC-NH_2

Scheme 4 – Tsuji-Trost of anti-HER2 affibody (11) with NBD derivative (12)

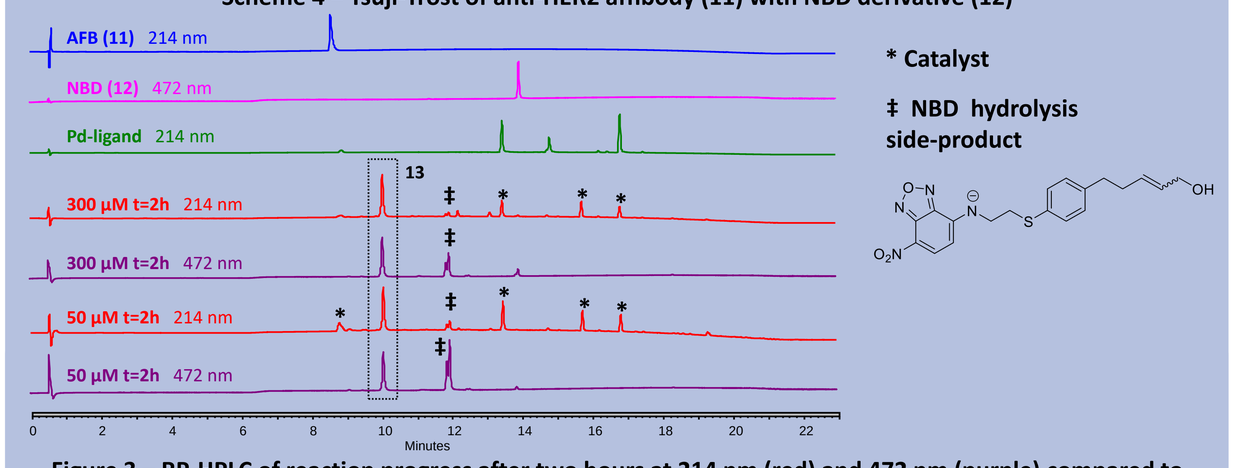


Figure 3 – RP-HPLC of reaction progress after two hours at 214 nm (red) and 472 nm (purple) compared to affibody 11 (blue), NBD derivative 12 (pink) and Pd-ligand solution (green) (15% MeCN in H_2O w/ 0.1% TFA over 5 min then 15 – 95% MeCN in H_2O with 0.1% TFA over 15 min)

At an affibody concentration of 300 µM, total conversion was achieved at 2 hours with **13** being obtained in a 71% yield following purification by semi-preparative RP-HPLC. As low as 50 µM, **13** was obtained in a good yield of 50% after 2 hours with almost complete conversion observed by RP-HPLC. Conversion was incomplete at 10 µM after 18 hours.

Future work and perspectives

- Full biological evaluation of KP-10 analogues (**5** – **7**) against G-protein coupled receptor 54, compared to native KP-10.
- Expansion of late stage diversification to include other thiol sources (ethyl-, isopropyl-) and oxidation to sulfones allowing facile access to a large library of compounds
- Demonstrate the ability to utilise the methodology for isotopic labelling (^{14}C) to access radiolabelled analogues for pharmacokinetic and pharmacodynamics studies
- Exploiting NBD fluorescence for cellular internalization assays of compound **13**
- Dual labelling of peptide/protein using sequential BHM/Tsuji Trost system

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