ACS Medicinal Chemistry Letters

Letter

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Direct Arginine Modification in Native Peptides and Application to Chemical Probe Development

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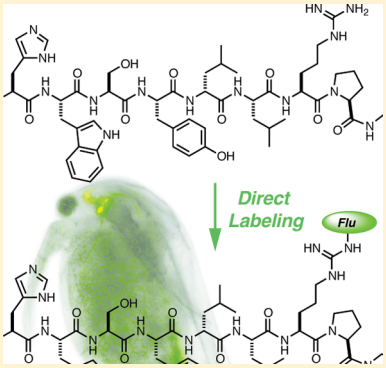
Supporting Information

ABSTRACT: An efficient method for the direct labeling of the Arg guanidinium group in native peptides is reported. This straightforward procedure allows modifying the arginine moiety in peptides with various reporter groups, such as fluorophores, biotin, etc., under mild conditions in an operationally simple procedure. The scope of this method tolerates various functionalized amino acids such as His, Ser, Trp, Tyr, Glu, etc., while the only limitations uncovered so far are restricted to cysteine and free amine residues. The utility of this late-stage diversification method was demonstrated in direct labeling of leuprolide, a clinically used drug, for distribution monitoring in *Daphnia*, and in labeling of microcystin, a cyanobacterial toxin.



KEYWORDS: *Chemical biology, peptide drugs, drug modification, drug mechanism of action*

The renaissance of peptides as key compounds in a variety of fields ranging from drug discovery and chemical biology to medical applications[1,2] has been fueled by improved methods for their synthesis.[3−7] In particular, further chemical elaboration of recombinantly produced peptides or their *de novo* synthesis have greatly expanded the reservoir of peptide backbone structures.[3−7] In addition, side chains of natural peptides and proteins are often post-translationally modified,[8−11] which require the development of efficient methods for side-chain functionalization.[3−7,12−18] Current methods typically rely on chemical modification at the NH_2, SH, or OH groups of Lys, Cys, Ser, Thr, Glx, or Asx residues.[3−7,12−18] In contrast, direct labeling of the Arg side chain is much less developed,[19−29] which is mainly due to the high pK_a value of the guanidinium group (12.5). While several methods for arginine side-chain modification via acylation of the guanidine functionality have been described in the literature, these methods are mainly suited for modification of arginine alone, rather than functionalization of arginine-containing peptides.[19−29] New methods allowing the direct modification of more complex, native peptides would therefore be highly desirable. One direct procedure for arginine side-chain modification is the glyoxal approach.[24,25,29] Unfortunately, this strategy is characterized by sensitivity of the intermediates and compromised yields. An expansion of this method, the use of 1,3-diketones,[23,27,28] suffers from long reaction times, low conversion, and consequently low yields. Another widely used method employs sodium or potassium hydroxide to deprotonate the guanidinium group to increase its nucleophilicity.[26] Such strongly basic conditions, however, are not compatible with some sensitive peptides and result in side reactions such as isomerization and degradation. The use of NEt_3, DBU, or Hünig's base represents an alternative to hydroxide bases. However, for some more complex systems, especially on a small synthetic scale, those methods often result in poor reactivity and therefore low yields.[19,20,22] In the context of our research program on unusual peptide natural products[30−34] and fluorescent natural product probes,[35] we became interested in addressing this problem.

We report here an efficient and direct method for modifying the arginine side-chain in peptides by an operationally simple procedure. The method makes use of the innate reactivity of the guanidinium group and matches its reactivity by the use of Barton's base, which ensures complete deprotonation and efficient subsequent nucleophilic attack on the acylating agent. Using this methodology, free arginine, as well as a number of peptides bearing unprotected side chains have been successfully tagged with a variety of relevant labels (fluorescent moieties, diazirine, and biotin). In addition, we present an application of this method by direct labeling of Arg in unprotected leuprolide, a clinically used drug. The environmental fate of this labeled probe was studied in the ecotoxicologically relevant organism *Daphnia magna*. The scope of this method was further demonstrated by the labeling of microcystin, a naturally occurring toxin, with biotin and diazirin groups.

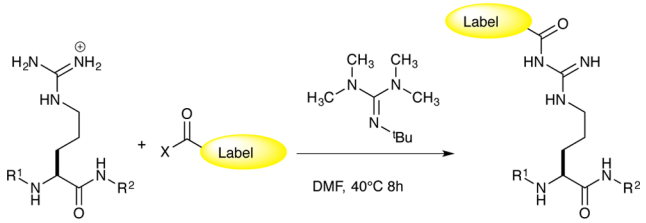
We first set out to identify and develop a suitable method in which Ac−Arg−OH served as a test substrate. Screening of a variety of basic reagents (both organic bases such as pyridine derivatives and tertiary amines or inorganic bases such as

Received: August 29, 2014
Accepted: October 27, 2014
Published: October 27, 2014

dx.doi.org/10.1021/ml5003508 | *ACS Med. Chem. Lett.* 2014, 5, 1290−1295

hydroxides and carbonates, or different coupling agents) led to the identification of 2-(*tert*-butyl)-1,1,3,3-tetramethylguanidine (Barton's base) as an ideal proton acceptor. The use of this commercially available base, which is characterized by poor nucleophilicity and a pK_a value of 15.3 (for the corresponding acid),[36] assures complete and fast deprotonation of the Arg guanidinium group, therefore enabling nucleophilic attack of the activated esters (Scheme 1).

Scheme 1. Direct Labelling of Arginine Containing Peptides



After careful experimentation, we identified the use of either *N*-hydroxysuccinidyl- or *p*-nitrophenylesters (1.5 equiv) in DMF as solvent at 40 °C as optimal conditions, which often led to complete reactions within 1 h (as judged by HPLC analysis of the reaction mixture). We then wanted to investigate the scope and limitations of this method to address the range of compatible functional groups. Therefore, a series of penta- to hepta-peptides containing various functionalized amino acids (Ser, Glu, Lys, Cys, His, etc.) were synthesized by standard solid-phase methodology (see Supporting Information for details).

We first subjected the synthesized peptides to acylation with 5(6)-carboxyfluorescein succinimide under optimized conditions. The reaction was performed by dissolving the peptides in dry DMF and then adding Barton's base to the solution. Subsequently, the mixture was stirred for 15 min to ensure complete deprotonation of the guanidinium group followed by dropwise addition of the activated ester. After the reaction was stirred for 1 to 8 h at 40 °C until completion, all volatiles were removed under reduced pressure, and the remaining residue was dissolved in water and directly purified by RP-HPLC to give the labeled peptides in moderate to good yield (>95% purity). A pentapeptide carrying all "innocent", hydrophobic side chains was modified to give acylated peptide **1** (Figure 1).

Peptides with either a Glu residue (to give compound **2**) or a Ser residue (as in **3**) were prepared, and acylation on the guanidinium was found to be preferred over both CO_2H and OH groups. It should be pointed out that all peptides were obtained with free *C*-termini, i.e., carboxylate groups, and that modification was never observed directly at the C-terminus. Experimental evidence for the regioselective Arg labeling was provided by MS/MS experiments that support the presence of labeled fragments (see Supporting Information). Likewise, the presence of a His residue in peptide **4** was also well tolerated, and the nucleophilic imidazole ring did not result in an incompatibility with the desired Arg modification. The existence of multiple Arg residues did not hamper the method, and increasing the corresponding equivalents of base allowed for smooth conversion to the bis-labeled peptide **5**. In addition, longer peptides such as heptapeptide **6** was successfully labeled, as was the peptide **7** carrying the nonproteinogenic Dha (didehydro alanine) amino acid.[37] This is of special interest, as the presence of the α,β-unsaturated acceptor could interfere with the various nucleophiles in the reaction.[38] In addition, these amino acids are frequently observed in peptides of biological interest such as microcystin (see below). Among the limitations encountered, we observed that tagging of Arg has so far not been possible in the presence of Lys or Cys residues, as their side chains interfere with the acylating agent. These limitations could be overcome by a transient protection, i.e., as an imine for Lys and a disulfide for Cys, which would then be *in situ* removed. In addition, it should be pointed out that in precursor peptides for native chemical ligation, Cys residues are often masked.[39]

We next evaluated the scope of the acylating agents, both with regard to the nature of the activated carboxylate derivative as well as concerning the nature of the group for conjugation. While the studies for peptides **1**–**9** have been carried out using *N*-hydroxysuccinimide esters, we next evaluated the use of *p*-nitrophenylesters. To this goal, the corresponding biotin derivatives were prepared and subjected to the identical reaction conditions as described above. Pleasingly, the corresponding peptides **10** and **11** biotinylated at the Arg residue were obtained in similar yields as for the fluorescent derivatives. For a third class of molecular probes, the sensitive diazirin-tag was successfully introduced using this method, and the peptides **12** and **13** bearing a photoaffinity label at the Arg position were prepared.

We then sought to apply this method for the fluorescent labeling of a bioactive, clinically used peptide. Direct labeling such widely used peptide drugs could allow the monitoring of their distribution, both within the patient and also in environmental samples. For this purpose, we selected the commercially available and clinically used nonapeptide, leuprolide acetate (leuprorelin, **14**).[40] This highly biologically active peptide constitutes an agonist of the luteinizing hormone-releasing hormone receptor and is in clinical use for a variety of indications related to the suppression of luteinizing hormone, in particular prostate cancer, endometriosis, central precocious puberty, and *in vitro* fertilization techniques.[40] Recent experimental evidence suggests that leuprolide possesses neurotrophic properties,[41] which is of interest in the context of our research program on small molecule neurotrophin mimics.[42,43] From a chemical point of view, leuprolide (**14**) contains a variety of functionalized amino acids that could interfere with the acylation protocol developed herein (Scheme 2).

Using the method described above, we were able to selectively acylate leuprolide (**14**) at the guanidinium side chain of arginine, and the fluorescently labeled leuprolide analogue **15** was obtained after HPLC purification in high purity (>95%) and good yield (77%). It is noteworthy that the presence of pGlu, His, Trp, Ser, and Tyr residues bearing several nucleophilic groups did not interfere with Arg labeling, which underlines the utility and selectivity of the method presented herein.

In order to study the environmental fate of leuprolide derivative **15**, we chose *Daphnia magna* as a test organism, which is a small grazing crustacean used as a model in ecotoxicology worldwide.[44] The labeled peptide **15** was dissolved in the medium, and the animals were subsequently exposed for 3 and 6 h, respectively. The living animals were washed and immobilized in 1% agarose gel for microscope analysis using a confocal microscope. As can be seen in the whole body image (Figure 2A), the labeled compound **15** has entered the digestive tract of the animal. More interestingly,

dx.doi.org/10.1021/ml5003508 | *ACS Med. Chem. Lett.* 2014, 5, 1290–1295

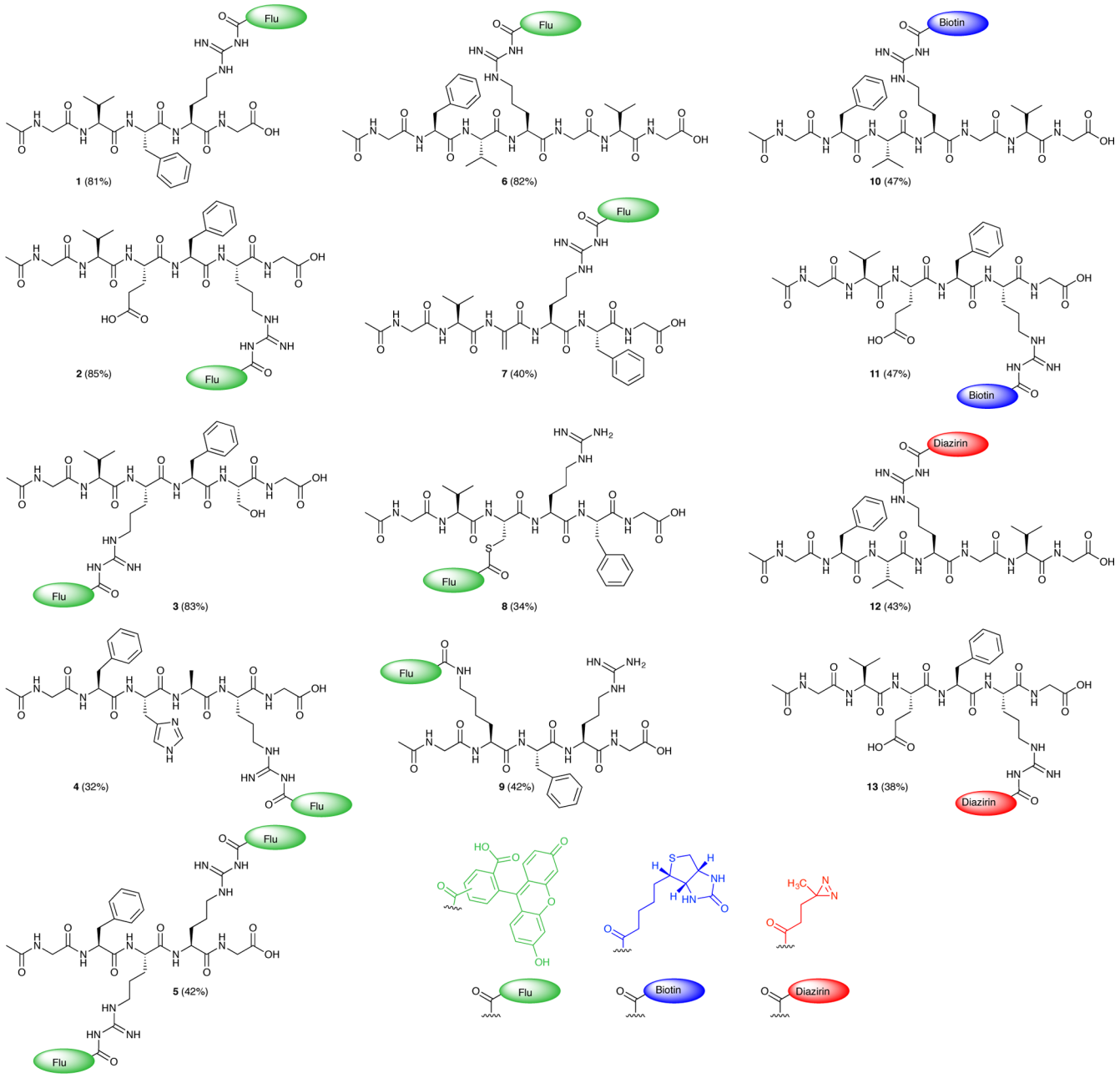


Figure 1. Various peptides labeled with carboxyfluorescein (green), biotin (blue), or diazirin (red) tags.

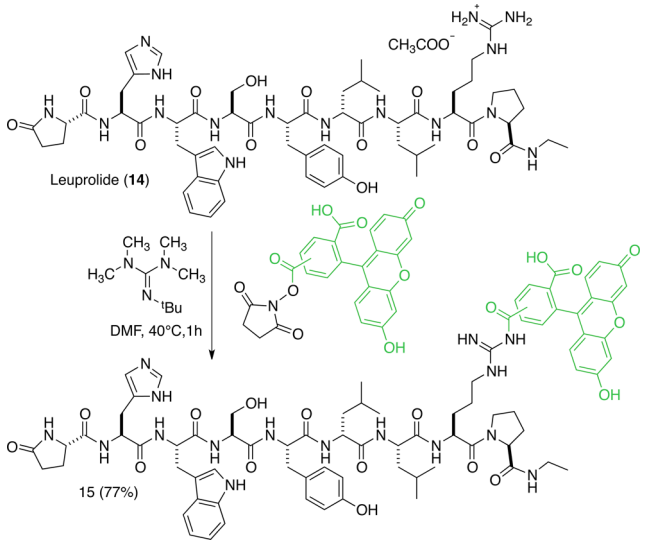
uptake into the cells of the ventral ceca (hepatopancreas)[45] has occurred (Figure 2B), which requires passing of the peritrophic membrane.

In addition to the labeling of the clinically used drug leuprolide, we sought to demonstrate the usefulness in the context of the derivatization of microcystin. This nonribosomal peptide features a number of nonproteinogenic amino acids and constitutes an abundant toxin in freshwater bodies, with threshold limits set by the world health organization for its ingestion in drinking water.[46] We have been able, using the method documented in this study, to prepare two chemical probes derived from microcystin, and the biotinylated compound **16** as well as the diazirin probe **17** have been obtained (Figure 3). Their successful synthesis allows for the generation of suitable microcystin probes for a number of applications in chemical biology.

The method documented in this study furnishes *N*-acyl guanidine derivatives, which warrant a number of comments: (1) The products **1**−**13** and **15**−**17** are chemically stable, can be purified by standard reversed phase HPLC methods, and are stable in normal buffer media for subsequent biological studies; (2) a search of the chemical literature reveals around 3000 *N*-acyl guanidine derivatives documented; and (3) the *N*-acyl guanidinium unit is found in a variety of natural products such as, e.g., bistellettadines, argadin, and banyasin A.[47] In addition over the last years, a large body of experimental evidence demonstrated the occurrence of various post-translational modifications at the guanidine group of Arg beyond methylation.[48] In this regard, the method developed in this work could allow for an access to some of these modifications found in natural peptides[47,49,50] and proteins.

dx.doi.org/10.1021/ml5003508 | *ACS Med. Chem. Lett.* 2014, 5, 1290−1295

Scheme 2. Direct and Selective Labelling of Leuprolide (14) with a Fluorescent Derivative to Give Derivative 15



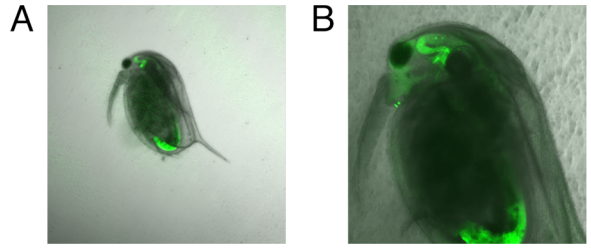


Figure 2. Exposure of *D. magna* to fluorescently labeled leuprolide derivative **15**.

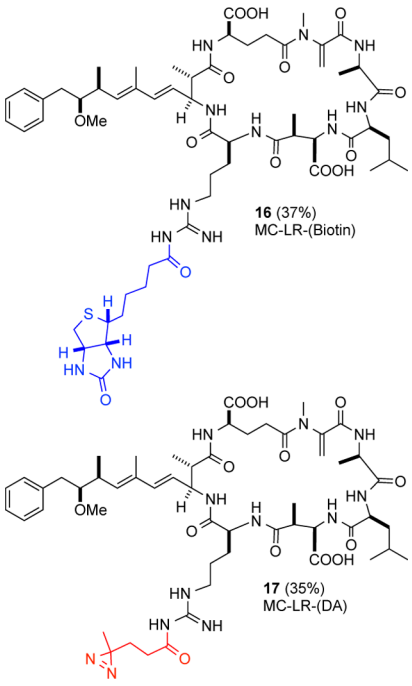


Figure 3. MC-LR labeled with biotin (blue) or diazirin (red) tags.

In conclusion, we have developed a method for the direct labeling of the guanidinium side chain of arginine in native peptides. The method relies on the use of Barton's base, which matches the innate reactivity of the guanidinium group in the substrate with a similar chemical structure of higher basicity. A series of peptides featuring Glu, Ser, His, Trp, Tyr, or Dha residues carrying sensitive side-chains has been selectively acylated at the Arg residue using both *N*-hydroxysuccinimide and *p*-nitrophenyl esters with fluorescent, diazirin, and biotin labels. As two direct applications of this method, the clinically used nonapeptide leuprolide was selectively acylated at the Arg residue, as well as the cyanobacterial toxin microcystin. We think that this method will find applications in biochemical, toxicological, pharmacological, and environmental studies of Arg-labeled peptides.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details for the synthesis, along with characterization and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the Swiss National Science Foundation in the context of the ProDoc program "Predictive Toxicology" (PDFMP3_132466).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. C. Sparr for critical reading of the manuscript, K. Tishinov for providing the biotin *p*-nitrophenylester, and J. Gao for providing the 2,5-dibromohexanediamide. Furthermore, we would like to thank S. Faltermann for her support with the uptake studies in *Daphnia magna*. We thank Prof. Dr. D. Ebert (University of Basel) for discussions on *D. magna*.

■ ABBREVIATIONS

RP-HPLC, reverse-phase high-performance liquid chromatography; MC-LR, microcystin-LR; DA, diazirin

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on October 31, 2014, with an error in the abstract graphic, Scheme 2, and in the Supporting Information. These items were corrected in the version published ASAP on November 24, 2014.

dx.doi.org/10.1021/ml5003508 | *ACS Med. Chem. Lett.* 2014, 5, 1290−1295