Synthesis of β-Thiol Phenylalanine for Applications in One-Pot Ligation-Desulfurization Chemistry

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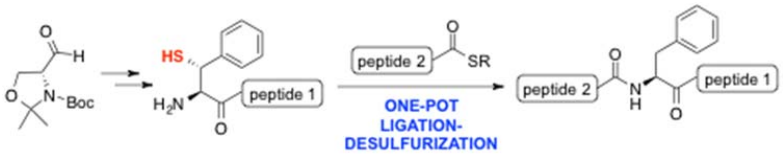
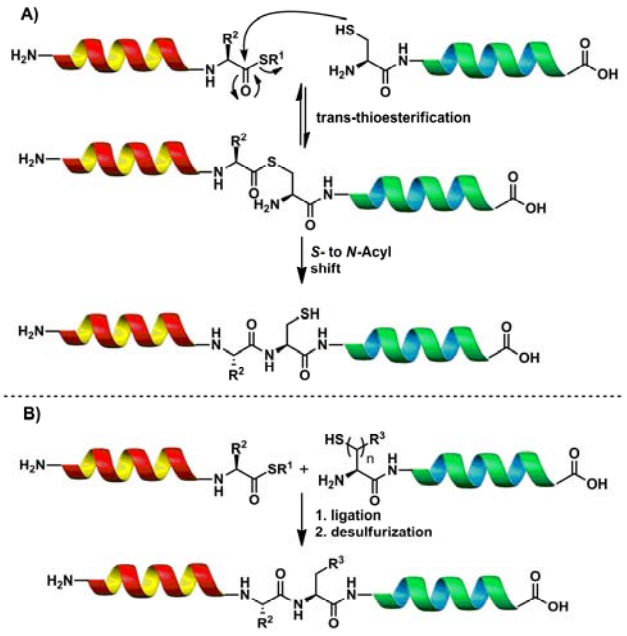
Supporting Information Placeholder



ABSTRACT: The efficient synthesis of a β-thiol phenylalanine derivative is described starting from Garner's aldehyde. The utility of this amino acid in peptide ligation-desulfurization chemistry is described, including the trifluoroethanethiol (TFET)-promoted one-pot assembly of the 62 residue peptide hormone augurin.

Twenty years following the original disclosure of the convergent assembly of unprotected peptide fragments,[1] native chemical ligation remains the most robust method for the synthetic preparation of protein targets.[2] The reaction, which takes place in aqueous media at neutral pH, involves a reversible trans-thioesterification step between a peptide containing an N-terminal cysteine (Cys) and a peptide bearing a C-terminal thioester functionality (Scheme 1A). This initial capture step is followed by a rapid, intramolecular S to N acyl shift to generate the native peptide bond. In recent years, significant research effort has focused on extending the scope of native chemical ligation-based transformations to enable ligation at residues other than Cys.[3] This concept was catalyzed by an initial report from Yan and Dawson,[4] which demonstrated that peptides and proteins produced *via* native chemical ligation could be desulfurized to provide an alanine (Ala) residue at the ligation junction. In the same report, the authors proposed the concept of further expanding the technology to other thiol-derivatized proteinogenic amino acids at the N-terminus of peptide fragments through the use of ligation-desulfurization chemistry (see Scheme 1B).[5] Since this early proposal, there has been a flourish of activity, especially in the last decade, that has led to successful syntheses of β-, γ- and δ-thiol amino acids,[6] including arginine (Arg),[7] aspartic acid (Asp),[8] glutamic acid (Glu),[9] glutamine (Gln),[10] phenylalanine (Phe),[11] valine (Val),[12] lysine (Lys),[13] leucine (Leu),[14] threonine (Thr)[15] and proline (Pro)[16] residues. These building blocks have also been successfully employed in the synthesis of a small number of protein targets to date.[3,5b,17]

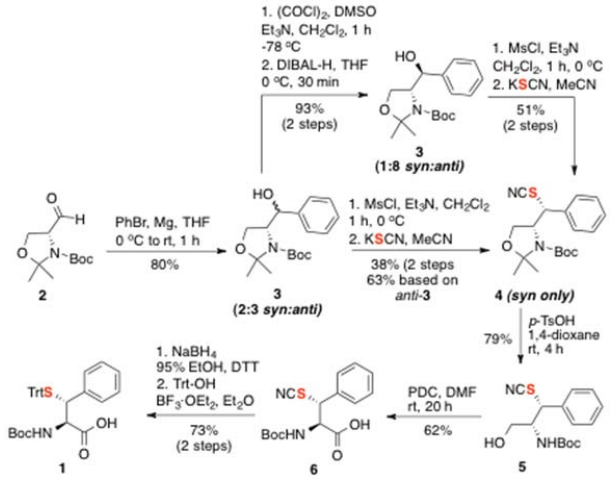
Although significant progress has been made to maximize the scope of native chemical ligation, synthetic access to suitably protected thiol-derived amino acid building blocks remains challenging. With the exception of two commercially available derivatives (penicillamine, a **β**-thiol surrogate of Val,[12a] and **γ**-thiol Pro[16a]) and our recent disclosure of peptide ligations promoted by the late-stage introduction of a 2-thiol tryptophan (Trp) auxiliary onto unprotected peptides,[18] most thiol-derived amino acids require multiple synthetic steps. Indeed, a general synthetic route to access a range of these important molecules does not currently exist. Applications of ligation-desulfurization technology at non-Cys junctions are therefore usually limited to the laboratories that developed the synthesis of a given thiol-derived amino acid.[17a]



Scheme 1. A) Mechanism of native chemical ligation B) Ligation-desulfurization at thiol-derived amino acids.

In seeking a robust and general synthetic strategy capable of delivering a wide range of thiol-derived amino acids, we have recently reported the use of Garner's aldehyde[19] as a common chiral starting point for the synthesis of suitably protected β-thiol Arg[7] and β-selenol Phe[20] building blocks. In principle,

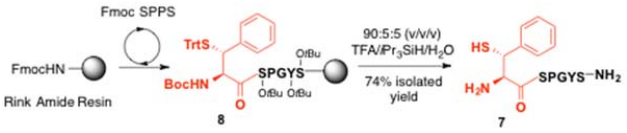
Garner's aldehyde can serve as a common starting point for the incorporation of most of the side chains present in proteinogenic amino acids as well as the incorporation of thiol or selenol reaction auxiliaries to enable ligation-desulfurization/deselenization chemistry[3,5b] at almost any amino acid. Herein, we report the efficient preparation of a suitably protected β-thiol Phe building block **1** from Garner's aldehyde (Scheme 2), with a view to further expanding the use of this chiral molecule as a common starting point to access a range of β-thiol amino acid derivatives. While β-thiol Phe has already been demonstrated to be a competent Cys surrogate for the ligation-based assembly of small peptides by Crich and Banerjee,[11a] in this study we aimed to shorten the synthesis of the amino acid and demonstrate the utility of the building block in both stepwise ligation-desulfurization chemistry and in efficient one-pot operations for the ligation-based assembly of larger peptide and protein targets.



Scheme 2. Synthesis of suitably protected β-thiol Phe (1) from Garner's aldehyde (2).

The synthesis of **1** began with a Grignard addition of bromobenzene to Garner's aldehyde **2** which gave alcohol **3** as a 2:3 mixture of *syn:anti* diastereoisomers in excellent yield (Scheme 2). Mesylation of diasteromeric **3** followed by displacement with potassium thiocyanate then provided **4** in 38% yield over the two steps. Importantly, only the *anti*-mesylate proved to be competent in the thiocyanate inversion reaction and thus provided the *syn*-thiocyanate exclusively. The corresponding *syn*-mesylate was recovered as unreacted starting material. These results are consistent with an analogous inversion performed with potassium selenocyanate for the synthesis of β-selenol Phe.[20] In order to improve the overall yield of the inversion, oxidation of **3** could be performed under Swern conditions followed by a DIBAL-H reduction to give *anti*-enriched **3** (1:8 *syn:anti*).[21] Mesylation and displacement with potassium thiocyanate then provided **4**, in 51% yield over the 2 steps, as the *syn*-diastereoisomer. From here, cleavage of the hemiaminal moiety in **4** using *p*-toluenesulfonic acid in 1,4-dioxane provided alcohol **5** in 79% yield. Oxidation of the primary alcohol with pyridinium dichromate then afforded carboxylic acid **6** in 62% yield. Finally, borohydride reduction of the thiocyanate followed by trityl (Trt) protection using Trt-OH in the presence of a Lewis acid afforded β-(trityl)thiol Phe derivative **1** in a total of 7 steps and 10.8% overall yield (for the oxidation-reduction pathway: 9 steps, 13.6% overall yield). These synthetic routes therefore represent efficient alternatives to the preparation of a β-thiol phenylalanine building block previously described by Crich and Banerjee (9 total steps from L-phenylalanine, 13.4% yield).[11a,22]

Model hexapeptide **7** was next prepared by Fmoc-strategy SPPS starting from Rink amide resin, whereby β-thiol Phe derivative **1** was incorporated at the N-terminus to afford resin-bound peptide **8** (Scheme 3). Cleavage from the resin and purification by reverse-phase HPLC provided peptide **7** in 74% yield based on the original resin loading (see Supporting Information for synthetic details). A variety of *C*-terminal peptide thioesters (Ac-LYRANX-S$(CH_2)_2CO_2$Et, X = Gly, Ala, Met, Phe, Val) were also prepared to study the scope of the ligation-desulfurization reactions with a range of amino acids on the C-terminus of the acyl donor component.



Scheme 3. SPPS of peptide 7 bearing an N-terminal β-thiol Phe residue.

With β-thiol peptide **7** and a variety of peptide thioesters now in hand, we next performed ligation reactions under standard native chemical ligation conditions [6 M guanidine hydrochloride (Gn•HCl), 100 mM Na_2HPO_4] in the presence of 50 mM TCEP and 2 vol % thiophenol at room temperature and a final pH of 7.2-7.4 (Table 1). In all cases, ligations proceeded in excellent yields (72-87%) and were complete within 24 h, including with the more sterically demanding Val thioester (entry 5). The ligation was also chemoselective in the presence of the ε-amino group of lysine (see Supporting Information for model ligation). Subsequent free-radical desulfurization in the presence of TCEP, 2,2'-azobis-[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044)[23] and glutathione[12a] at 65 °C cleanly provided the native Phe residue at the ligation junction affording native peptide products in 52-87% yields (Table 1).

We were also interested in comparing the rate of ligation at β-thiol Phe with that of native chemical ligation at Cys. To this end, we prepared the model N-terminal peptide H-CSPGYS-NH_2 and evaluated the rate of ligation with Ac-LYRANG-S$(CH_2)_2CO_2$Et (see Supporting Information) compared with the corresponding ligation of peptide **7**. To our surprise, ligation with β-thiol Phe-containing peptide **7** reached completion in less than 30 minutes, proving to be only modestly slower than ligation at the native Cys residue, despite the additional steric bulk associated with the phenyl side chain (see Supporting Information for kinetic data). In addition, we conducted a competition experiment between peptide **7** and a homologue bearing our N-terminal β-selenol Phe residue.[20] This reaction was carried out under conditions commonly employed for selenium-mediated ligations, namely in the absence of TCEP (which is known to facilitate deselenization)[24] and in the presence of 4-mercaptophenylacetic acid (MPAA) as both a thiol additive and mild reductant (see Supporting Information). Interestingly, the competitive ligation of peptide **7** (1.0 eq.) and the corresponding selenopeptide dimer (**S1**, 1.0 eq., see Supporting Information) with a substoichiometric amount of peptide thioester occurred to provide exclusively the thiol-Phe ligation product. As previously suggested,[20,25] it is postulated that the rate-determining step in

the selenol-mediated ligation, particularly in the absence of a strongly reducing phosphine (e.g. TCEP), is the generation of reactive selenol from the starting peptide, which exists in oxidized form as the diselenide dimer (see Supporting Information). Importantly, the observed rate differential suggests that kinetically-controlled ligation reactions employing both thiol-Phe and selenol-Phe may be feasible for the iterative assembly of target peptides and proteins from multiple fragments.

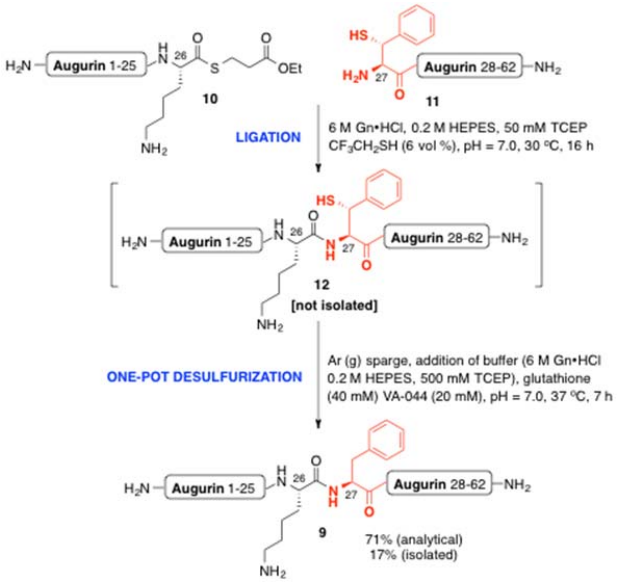
Table 1. β-thiol Phe ligation-desulfurization reactions

thioester (X =)	ligation yield[a]	desulfurization yield[a]	one-pot ligation-desulfurization yield[a]
Gly (G)	79%	87%	68%
Ala (A)	86%	76%	79%
Met (M)	72%	52%	71%
Phe (F)	83%	60%	87%
Val (V)	87%	67%	68%

[a]Isolated yields; ***Reaction conditions:*** **Ligation:** Thioester (1.1-1.3 eq., 5.5-6.5 mM concentration), buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 50 mM TCEP, 5 mM with respect to peptide **7**), 2 vol % thiophenol, 37 °C, pH 7.2-7.4, 24 h. **Desulfurization:** VA-044, buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 500 mM TCEP), 40 mM glutathione, 65 °C, 16 h. **One pot ligation-desulfurization:** Thioester (1.1-1.3 eq., 5.5-6.5 mM concentration), buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 50 mM TCEP, 5 mM final concentration with respect to peptide **7**), 2 vol % TFET, 30 °C, pH 7.0-7.4, 16 h; then degas (Ar), dilute with buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 500 mM TCEP, pH adjusted to 6.0) to a final concentration of 2.5 mM with respect to peptide **7**, addition of glutathione (40 mM), VA-044 (20 mM), 37 °C, 6-7 h.

We have recently reported the use of the alkyl thiol trifluoroethanethiol (TFET) as an additive for one-pot native chemical ligation-desulfurization reactions with Cys residues.[17d] Here, we were interested in employing TFET in one-pot ligation-desulfurization reactions at β-thiol Phe in order to streamline the methodology and reduce the number of intermediary purification steps (Table 1). To this end, peptide **7** was reacted under modified ligation conditions [6 M Gn•HCl, 100 mM Na_2HPO_4, 50 mM TCEP] in the presence of 2 vol % TFET at 30 °C and a final pH of 7.0-7.4. After 16 h, the reaction was sparged with argon and diluted with degassed buffer [6 M Gn•HCl, 100 mM Na_2HPO_4, 500 mM TCEP, pH adjusted to 6.0] before the addition of VA-044 and reduced glutathione to effect desulfurization of the β-thiol auxiliary in the ligation products. The desulfurization reactions were incubated for 6-7 h at 37 °C before purification by reverse-phase HPLC. Gratifyingly, products from these one-pot ligation-desulfurization reactions were isolated in 68-87% yield over the two steps (average of 82-93% per step).

Having demonstrated the efficiency of the one-pot ligation-desulfurization manifold at β-thiol Phe for model peptides, we were next interested in using this methodology for the construction of a more synthetically challenging target. Specifically, we selected as a demonstrative example a 62-amino acid fragment of the putative secreted peptide hormone augurin **9**, which is endoded by Esophageal Cancer Related Gene-4 (Ecrg4) and expressed in endocrine tissue but has a function that is as yet unknown.[26] It was envisaged that this Cys-free peptide target could be rapidly prepared using a TFET-promoted one-pot ligation-desulfurization at β-thiol Phe (Scheme 4). To this end, peptide thioester **10** (augurin 1-26), bearing a C-terminal Lys residue, and peptide **11** (augurin 27-62), bearing an N-terminal β-thiol Phe residue were first prepared using Fmoc-SPPS (see Supporting Information). Following purification of the requisite fragments, the ligation reaction was carried out in the presence of a slightly modified buffer solution (6 M Gn•HCl, 0.2 M HEPES, 50 mM TCEP, 6 vol % TFET), most notably in the absence of phosphate to minimize the potential for N-terminal pyroglutamate formation[27] at the terminal Gln residue of peptide thioester **10**. The pH of the reaction was also carefully controlled to minimize base-catalyzed lactamization of the C-terminal Lys-thioester moiety. After 16 h, the ligation was deemed to be complete *via* HPLC-MS analysis. The crude ligation product **12** was subjected directly (without intermediary purification) to the radical desulfurization conditions outlined above, cleanly affording the target product bearing a native Phe residue at the ligation junction. The efficiency of the one-pot protocol is reflected in the analytical yield of the ligation-desulfurization reaction (71% yield of product **9**, with the corresponding ligation product bearing an N-terminal pyroglutamate as a minor byproduct in 20% yield, see Supporting Information). Although the aggregation-prone nature of augurin[28] hindered the facile isolation of the target peptide, purified **9** was nonetheless obtained in 17% isolated yield. The rapid and efficient synthesis of this difficult peptide target showcases the utility of one-pot ligation-desulfurization reactions mediated by **β**-thiol Phe.



Scheme 4. One-pot synthesis of augurin 9.

In summary, we have developed a novel synthetic route to β-thiol Phe which highlights the generality of Garner's aldehyde as a common chiral precursor to both thiol- and selenol-derived amino acids. We have expanded the scope of ligation reactions at thiol Phe and explored the kinetics of the transformation for the first time. Moreover, we have demonstrated that ligation products can be desulfurized to provide native peptide products through a streamlined one-pot ligation-desulfurization approach employing the thiol additive TFET. The utility of this methodology was exemplified through the efficient, ligation-based assembly of the 62-amino acid peptide hormone augurin. Future work will focus on the use of β-thiol Phe in the synthesis of other complex protein targets. Further studies will also focus on the exploration of kinetically-controlled and tandem ligation reactions employing both β-thiol and β-selenol Phe derivatives.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, analytical HPLC traces and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.

(2) Kent, S. B. *Chem. Soc. Rev.* **2009**, *38*, 338.

(3) Malins, L. R.; Payne, R. J. *Curr. Opin. Chem. Biol.* **2014**, *22*, 70.

(4) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526.

(5) (a) Rohde, H.; Seitz, O. *Biopolymers* **2010**, *94*, 551; (b) Dawson, P. E. *Isr. J. Chem.* **2011**, *51*, 862; (c) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem. Int. Ed.* **2008**, *47*, 10030.

(6) Wong, C. T. T.; Tung, C. L.; Li, X. *Mol. Biosyst.* **2013**, *9*, 826.

(7) Malins, L. R.; Cergol, K. M.; Payne, R. J. *ChemBioChem* **2013**, *14*, 559.

(8) (a) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. *Angew. Chem. Int. Ed.* **2013**, *52*, 9723; (b) Guan, X.; Drake, M. R.; Tan, Z. *Org. Lett.* **2013**, *15*, 6128.

(9) Cergol, K. M.; Thompson, R. E.; Malins, L. R.; Turner, P.; Payne, R. J. *Org. Lett.* **2014**, *16*, 290.

(10) Siman, P.; Karthikeyan, S. V.; Brik, A. *Org. Lett.* **2012**, *14*, 1520.

(11) (a) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064; (b) Botti, P.; Tchertchian, S. **2006**, WO/2006/133962.

(12) (a) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem. Int. Ed.* **2008**, *47*, 6807; (b) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J. L.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2008**, *47*, 8521.

(13) (a) Yang, R. L.; Pasunooti, K. K.; Li, F. P.; Liu, X. W.; Liu, C. F. *J. Am. Chem. Soc.* **2009**, *131*, 13592; (b) Ajish Kumar, K. S.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. *Angew. Chem. Int. Ed.* **2009**, *48*, 8090; (c) El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H. *Angew. Chem. Int. Ed.* **2010**, *49*, 10149.

(14) (a) Harpaz, Z.; Siman, P.; Kumar, K. S. A.; Brik, A. *ChemBioChem* **2010**, *11*, 1232; (b) Tan, Z. P.; Shang, S. Y.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2010**, *49*, 9500.

(15) Chen, J.; Wang, P.; Zhu, J. L.; Wan, Q.; Danishefsky, S. J. *Tetrahedron* **2010**, *66*, 2277.

(16) (a) Shang, S. Y.; Tan, Z. P.; Dong, S. W.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2011**, *133*, 10784; (b) Townsend, S. D.; Tan, Z.; Dong, S.; Shang, S.; Brailsford, J. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 3912; (c) Ding, H.; Shigenaga, A.; Sato, K.; Morishita, K.; Otaka, A. *Org. Lett.* **2011**, *13*, 5588.

(17) (a) Malins, L. R.; Payne, R. J. *Aust. J. Chem.* **2015**, doi: 10.1071/CH14568; (b) Shang, S.; Tan, Z.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 5986; (c) Kumar, K. S.; Bavikar, S. N.; Spasser, L.; Moyal, T.; Ohayon, S.; Brik, A. *Angew. Chem. Int. Ed.* **2011**, *50*, 6137; (d) Thompson, R. E.; Liu, X.; Alonso-García, N.; Pereira, P. J. B.; Jolliffe, K. A.; Payne, R. J. *J. Am. Chem. Soc.* **2014**, *136*, 8161.

(18) Malins, L. R.; Cergol, K. M.; Payne, R. J. *Chem. Sci.* **2014**, *5*, 260.

(19) Garner, P. *Tetrahedron Lett.* **1984**, *25*, 5855.

(20) Malins, L. R.; Payne, R. J. *Org. Lett.* **2012**, *14*, 3142.

(21) Nishida, A.; Sorimachi, H.; Iwaida, M.; Matsumizu, M.; Kawate, T.; Nakagawa, M. *Synlett* **1998**, 389.

(22) Crich, D.; Banerjee, A. *J. Org. Chem.* **2006**, *71*, 7106.

(23) Wan, Q.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2007**, *46*, 9248.

(24) Metanis, N.; Keinan, E.; Dawson, P. E. *Angew. Chem. Int. Ed.* **2010**, *49*, 7049.

(25) Gieselman, M. D.; Xie, L.; van Der Donk, W. A. *Org. Lett.* **2001**, *3*, 1331.

(26) Mirabeau, O.; Perlas, E.; Severini, C.; Audero, E.; Gascuel, O.; Possenti, R.; Birney, E.; Rosenthal, N.; Gross, C. *Genome Res.* **2007**, *17*, 320.

(27) Khandke, K. M.; Fairwell, T.; Chait, B. T.; Manjula, B. N. *Int. J. Pept. Protein. Res.* **1989**, *34*, 118.

(28) Podvin, S.; Gonzalez, A.-M.; Miller, M. C.; Dang, X.; Botfield, H.; Donahue, J. E.; Kurabi, A.; Boissaud-Cooke, M.; Rossi, R.; Leadbeater, W. E.; Johanson, C. E.; Coimbra, R.; Stopa, E. G.; Eliceiri, B. P.; Baird, A. *PLoS ONE* **2011**, *6*, e24609.