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Synthetic α- and β-Ser-ADP-ribosylated Peptides Reveal α-Ser-ADPr as the Native Epimer

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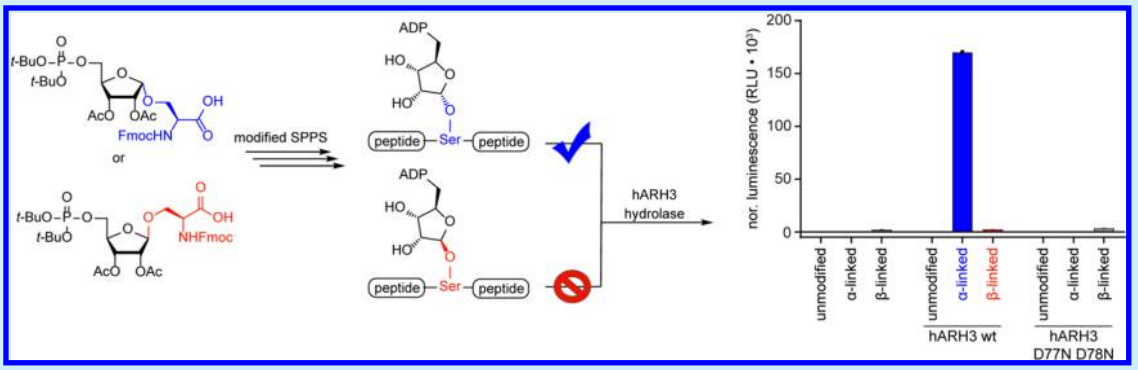
Supporting Information



ABSTRACT: A solid-phase methodology to synthesize oligopeptides, specifically incorporating serine residues linked to ADP-ribose (ADPr), is presented. Through the synthesis of both α- and β-anomers of the phosphoribosylated Fmoc-Ser building block and their usage in our modified solid-phase peptide synthesis protocol, both α- and β-ADPr peptides from a naturally Ser-ADPr containing H2B sequence were obtained. With these, and by digestion studies using the human glycohydrolase, ARH3 (hARH3), compelling evidence is obtained that the α-Ser-ADPr linkage comprises the naturally occurring configuration.

Post-translational modifications (PTMs) of proteins are involved in many biological processes and entail covalent and reversible modification of specific amino acid side chains.[1] Adenosine diphosphate ribosylation (ADP-ribosylation) is a PTM that has been associated with DNA damage response pathways, transcriptional regulation, aging, and apoptosis.[2] ADP-ribosylation is catalyzed by a series of enzymes known as adenosine ribosyltransferases (ARTs).[3] In this process, ARTs consume β-NAD$^+$ and transfer ADP-ribose (ADPr) onto nucleophilic side chains of various amino acids, including arginine, glutamate/aspartate, and serine.[4] This mono-ADP-ribosylated (MARylated) site can then be modified further by some ARTs forming long and also branched poly-ADPr (PAR) chains.

Recently, serine residues were shown to be both acceptors of ADPr[5] and the primary target of ADP-ribosylation in cells upon H_2O_2 treatment. Many of the proteins identified as Ser-ADP-ribosylated are critical for the maintenance of genome stability.[6,7] Moreover, amino acid selectivity in this pathway appears to be dependent on a switch of the catalytic preference of the ARTs PARP1 and PARP2 (also known as ARTD1 and ARTD2) from glutamate/aspartate toward serine residues, a feature effected by the formation of a newly identified PARP1-HPF1 complex.[6,8] Transfer of ADPr by PARP1 to Glu and Asp residues involves stereochemical inversion at the anomeric carbon and results in the formation of α-glycosidic linkages as the sole products of the reaction.[9] In contrast, the catalytic mechanism of the PARP1−HPF1 complex and the configuration of the resulting product remains unclear. Mechanistic studies of this complex as well as other ADP-ribosylation systems have been restrained by the unavailability of sufficient quantities of well-defined fragments of Ser-ADPr containing peptides. We here describe the preparation of such Ser-ADPr peptides from phosphoribosylated Fmoc-serine building blocks designed specifically for this purpose. These building blocks were applied in the solid phase approach previously described by us for the synthesis of ADPr-peptides that contained Gln-, Asn-, or Cit-ADPr,[10] which are close isosteres of the native Glu-, Asp-, Arg-ADPr linkages. In this way we prepared an *N*-terminal H2B peptide that contains Ser10, which is a confirmed in vivo ADPr acceptor site.[5,7] Preparation of the Ser-ADPr H2B peptide allowed us to unambiguously determine the anomeric configuration of the ribose residue and thus to gain the first mechanistic insights into the Ser-ADPr biology.

We prepared the Ser-ADPr H2B peptide in both the α- and β-anomeric state and treated these with the human ADP-ribosylhydrolase 3 (hARH3), the only known enzyme to

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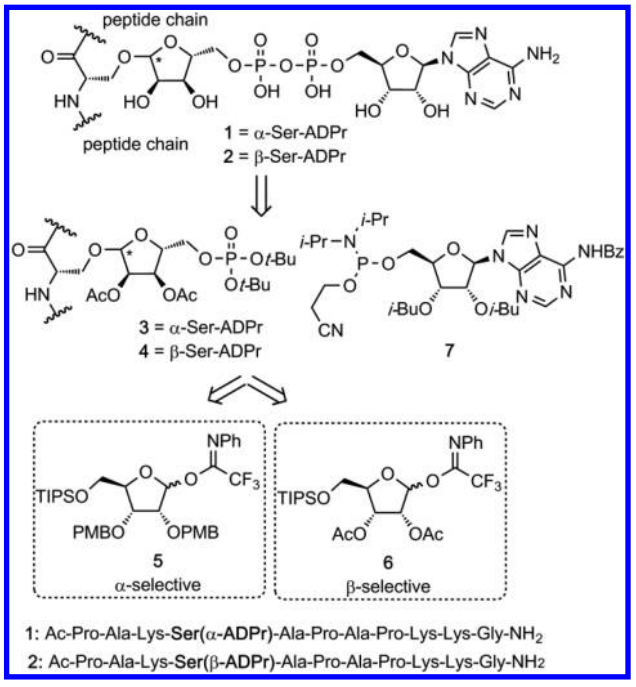
reverse Ser-ADPr.[11] The analysis revealed that only the α-anomer of Ser-ADPr could be enzymatically hydrolyzed, strongly suggesting that the *O*-glycosidic linkage formed by the PARP1-HPF1 complex is α-configured. Subsequent analysis of the chemical stability of the native α-Ser-ADPr peptide under conditions regularly encountered in biochemical and proteomics studies (includ ing aqueous acid or base and hydroxylamine) revealed the sensitivity of Ser-ADPr toward alkaline conditions. This finding has important implications for the proteomics studies of ADP-ribosylomes.[4,12]

As shown in Scheme 1, our retrosynthetic analysis of the target mono-ADP-ribosylated peptides **1** and **2** relies on ribose

Scheme 1. Retrosynthetic Analysis for the Synthesis of Mono-ADP-ribosylated Peptides 1 and 2 and Essential Building Blocks

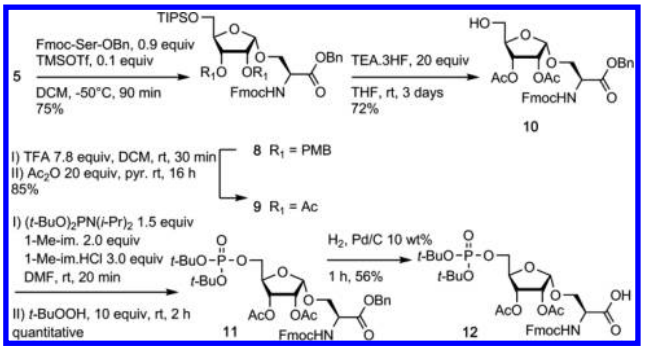


donors **5** and **6** for the stereoselective glycosylation of Fmoc-Ser-OBn. Acetyl protecting groups in α- and β-ribosylated serine building blocks were chosen to allow for orthogonal deprotection of the C-terminus in the final stage of the preparation of these building blocks. The phosphate was protected with *t*-Bu groups to enable the on-resin pyrophosphate formation via a procedure developed by us, which utilizes adenosine 5′-phosphoramidite **7** as the key phosphorylating agent.[8]

We previously reported *N*-(phenyl)trifluoroacetimidate **5** (Scheme 1) for the α-selective ribosylation of the side chain of various amino acids.[13] To obtain a β-selective donor, we replaced the *p*-methoxybenzyl (PMB) by acetyl (Ac) groups, giving 2,3-*O*-diacetylated *N*-(phenyl)trifluoroacetimidate **6** (Scheme 1). The preparation of **6** could be accomplished in 70% yield (Scheme S1) via a three-step sequence from known[12] allyl 5-O-triisopropylsilyl-α,β-D-ribofuranoside (**1S**, Scheme S1).

Having prepared the required ribofuranosyl donors, we first undertook an α-selective glycosylation of Fmoc-Ser-OBn with known[12] *N*-(phenyl)trifluoroacetimidate donor **5** (Scheme 2). When 0.2 equiv of TMSOTf was employed at −50 °C, α-

Scheme 2. Synthesis of Orthogonally Protected α-Phosphoribosylated Serine



configured ribosylated serine **8** was isolated in 75% yield, while the corresponding β-product could not be detected. Subsequent cleavage of the PMB protecting groups in **8** using our previously reported HCl/HFIP cocktail[14] (0.1 M) gave unsatisfactory yields due to acid-mediated transacetalization of the serine moiety. Although lowering the concentration of HCl (up to 0.01 M) in the mixture reduces this anomeric cleavage, scaling up of the reaction resulted again in moderate yields. Fortunately, treatment of **8** with TFA in DCM showed no sign of hydrolysis and upscaling of the reaction combined with the subsequent acetylation of the crude intermediate gave **9** in reproducible yields that exceeded 80% over two steps. Removal of the silyl ether in **9** using TEA·3HF proceeded uneventfully, while treatment with pyr·HF was accompanied by Fmoc cleavage. Phosphitylation of the primary hydroxyl in **9** using di-*tert*-butyl *N*,*N*-diisopropylphosphoramidite, and subsequent oxidation of the phosphite intermediate yielded phosphotriester **11**. Finally, hydrogenolysis of the benzyl ester gave orthogonally protected, α-phosphoribosylated serine building block **12**.

For the β-selective glycosylation of Fmoc-Ser-OBn with *N*-phenyltrifluoroacetimidate donor **6** different reaction conditions proved to be necessary. The conditions that were used for α-selective glycosylation (−50 °C, 0.2 equiv of activator) gave a low glycosylation yield and a significant amount of acetyl migration to the serine aglycon.[15] Therefore, optimization of the β-selective glycosylation reaction with respect to the donor/activator ratio and the reaction temperature was performed (Table S1). Eventually acceptable conditions that provided a 55% yield at the glycosylation step were found (Table S1, entry 4). These conditions (0 °C with 0.1 equiv TMSOTf) were selected for the β-selective glycosylation of Fmoc-Ser-OBn with donor **6**. Thus, the route to β-phosphoribosylated serine building block **14** started by condensation of donor **6** with Fmoc-Ser-OBn followed by immediate treatment of this crude mixture with TEA·3HF, to produce **13** in 44% yield over the two steps after a straightforward purification step (Scheme 3). Analogous to the synthesis of α-phosphoribosylated serine, standard phosphitylation of building block **13**, ensuing oxidation, and finally, cleavage of the benzyl ester gave β-phosphoribosylated building block **14**.

With building blocks **12** or **14** available, we synthesized hendecapeptides **1** and **2** (Scheme 1) on solid phase. The solid-phase peptide synthesis (Scheme S2) was done according to the protocol developed by us previously for the ADP-ribosylated peptides of a different nature.[8]

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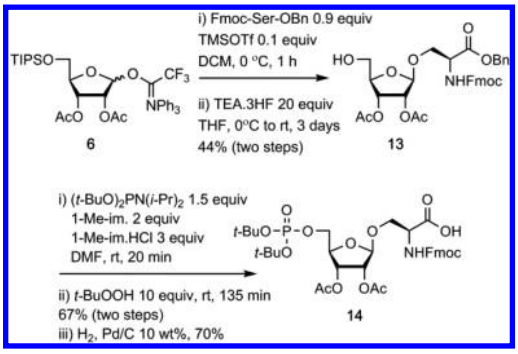
Scheme 3. Synthesis of the β-Phosphoribosylated Serine Building Block



Having obtained Ser-ADPr peptides **1** and **2**, we set out to establish the native stereochemistry at the anomeric center of the ribose residue attached to the side chain of serine. Human ARH3 is the only enzyme known to hydrolyze the *O*-glycosidic serine-ADPr linkage.[11] A homologue of ARH3, termed ARH1, is a stereoselective ADP-ribosylarginine hydrolase,[16,17] which led us to assume that ARH3 is likewise only capable of hydrolizing glycosidic linkages with the native stereochemistry, leaving the non-natural epimer intact. We performed a de-MARylation assay by treating the homogeneous α- and β-configured H2B peptides **1** and **2**, respectively, with hARH3. In addition, the reaction mixture contained human NUDT5 (hNUDT5), which converts the released ADPr into AMP, and thus allows detection of the peptide turnover by a commercial assay kit (see the Supporting Information for details). As expected, no turnover was observed either by hNUDT5 alone[18] or in the presence of the catalytically inactive hARH3 double mutant (D77N D78N) (Figure 1). On the other hand, wild-type hARH3 exclusively hydrolyzes the α anomeric serine-ADPr linkage. Since we did not observe spontaneous epimerization in solution, these data strongly suggest that the α-linked form is the endogenous epimer.

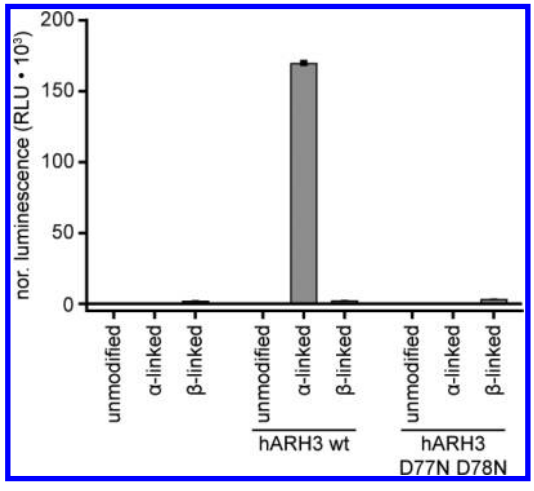
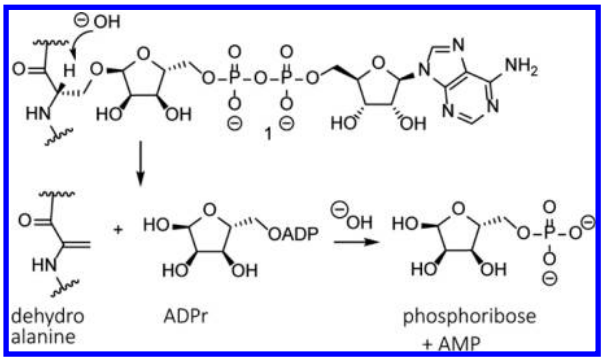


Figure 1. Analysis of the stereospecificity of hARH3. ADPr released in the ARH3 reaction was converted into AMP using hNUDT5 and subsequently measured using the AMP-Glo assay (Promega). Control reactions were carried out both in absence of hARH3 as well as using a catalytically inactive hARH3 mutant (D77N D788N). The data were normalized to reactions containing only hNUDT5 and represent triplicate measurement ± SD.

After confirming the stereochemistry of the naturally occurring Ser-ADPr modification, we tested the chemical stability of the native α-Ser-ADPr. Such knowledge about the stability of ADP-ribsylation sites is relevant for the future synthetic and proteomics studies and for a retrospective evaluation of already documented observations. We opted for a set of chemically divergent conditions (aqueous acid, diluted NaOH and 0.5 M NH_2OH) as these are broadly encountered in proteomic studies for identifying PAR- and MARylation sites[17,19] (NH_2OH, NaOH) or in the course of sample preparation (aqueous acid) (Scheme 4).[4] Peptide **1** was

Scheme 4. Mechanism of ADPr Elimination by NaOH



dissolved in aqueous solutions of either 0.1 M TFA, 0.1 M NaOH, or 0.5 M NH_2OH, and after various time points (15 min, 60 min, 2.5 h, 5 h, and 24 h), a sample was taken for LC–MS analysis of the quality of the peptide. For TFA and NH_2OH, no detectable degradation was observed after 24 h. This leads to the conclusion that the isolation and purification of Ser-ADPr-peptides can safely be performed under acidic conditions and that the treatment of ADPr-proteins with NH_2OH is indeed selective toward Asp and Glu residues since Ser-ADPr is unreactive toward this nucleophile.[19] However, treatment of our MARylated peptide **1** with 0.1 M aqueous NaOH (pH 13) showed a measurable elimination of ADP-ribose after 15 min (7%, Table 1). After 2.5 h, the eliminated

Table 1. Reaction of Ser-ADPr Peptide 1 with Aqueous NaOH

time (h)	intact ADPr peptide (%)	free ADPr (%)	free AMP (%)
0	100	0	0
0.25	93	7	0
1	85	15	0
2.5	66	26	8
5	45	32	23
24	0	50	50

ADPr was measurably hydrolyzed, giving phosphoribose and adenosine monophosphate (AMP). In a time span of 5 h, more than half of the material was eliminated into a mixture of ADPr or AMP and complete degradation ensued after 24 h. This elimination reaction leads to transformation of Ser-ADPr into dehydroalanine at the modification site, a reaction not possible with amino acids such as Asp, Glu, or Arg. This result is in line with the known behavior of serine *O*-glycopeptides, in which the glycosyl linkage also cleaved by β-elimination upon treatment with base.[20] The formation of dehydroalanine residue has been exploited in studies on phosphoproteomics[21,22] and glycomics,[23] and our observation that Ser-ADPr

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sites behave similarly points to a possibility of extending such "footprinting" to ADPr-ribosylome as well.

In conclusion, we have synthesized suitably protected phosphoribosylated serine building blocks and used these for the SPPS-mediated assembly of H2B-derived peptides, MARylated on their serine residues both in α- and β-glycosidic form, via our SPPS based strategy. The method promises to open up the possibility of synthesizing a wider range of Ser-ADPr peptides chemically and, following a native chemical ligation strategy, perhaps also full-length Ser-ADPr proteins. Thus, well-defined oligopeptides MARylated on serine and varying in composition should become accessible in sufficient amounts for biological studies. The H2B-derived peptides prepared in this study were used to investigate fundamental, but as yet unknown, properties of the Ser-ADPr modification. We utilized the striking specificity of ARH3 for Ser-ADPr substrates to gain insight into its substrate preference and by extension the stereoselectivity by which the PARP1−HPF1 complex catalyzes MARylation of serine residues. ARH3 catalysis resulted only in the hydrolysis of the α-linked peptide, thus strongly implying that this is the natural epimer. While further studies should address the exact catalytic mechanism of the complex, we hypothesize that HPF1 contributes mainly to target selection, rather than being actively involved in the ADPr transfer. Since the serine *O*-ribosidic bond is chemically distinct from the formerly studied Asp, Glu, and Arg modifications, we have tested the stability of this linkage under conditions typically encounter in biochemical and proteomic studies. Ser-ADPr proved to be stable for a minimum of 24 h when subjected to TFA (0.1 M) and NH_2OH (0.5 M) with no detectable degradation. In contrast, when Ser-ADPr was treated with NaOH (0.1 M) β-elimination occurred, in which the glycosidic bond was cleaved to give dehydroalanine residue and free ADPr.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b01742.

Synthesis of donors **5** and **6**, optimization of β-selective glycosylation with donor **6**, solid-phase peptide synthesis of **1** and **2**, experimental procedures, and copies of NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Lüscher, B.; Bütepage, M.; Eckei, L.; Krieg, S.; Verheugd, P.; Shilton, B. H. *Chem. Rev.* **2018**, *118*, 1092−1136.
(2) Gibson, B. A.; Kraus, W. L. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 411−424.
(3) Hottiger, M. O.; Hassa, P. O.; Lüscher, B.; Schüler, H.; Koch-Nolte, F. *Trends Biochem. Sci.* **2010**, *35*, 208−219.
(4) Daniels, C. M.; Ong, S.; Leung, A. K. L. *Mol. Cell* **2015**, *58*, 911−924.
(5) Leidecker, O.; Bonfiglio, J. J.; Colby, T.; Zhang, Q.; Atanassov, I.; Zaja, R.; Palazzo, L.; Stockum, A.; Ahel, I.; Matic, I. *Nat. Chem. Biol.* **2016**, *12*, 998−1000.
(6) Bonfiglio, J. J.; Fontana, P.; Zhang, Q.; Colby, T.; Gibbs-Seymour, I.; Atanassov, I.; Bartlett, E.; Zaja, R.; Ahel, I.; Matic, I. *Mol. Cell* **2017**, *65*, 932−940.
(7) Palazzo, L.; Leidecker, O.; Prokhorova, E.; Dauben, H.; Matic, I.; Ahel, I. *eLife* **2018**, *7*, e34334.
(8) Gibbs-Seymour, I.; Fontana, P.; Rack, J. G. M.; Ahel, I. *Mol. Cell* **2016**, *62*, 432−442.
(9) Morgan, R. K.; Cohen, M. S. *ACS Chem. Biol.* **2015**, *10*, 1778−1784.
(10) Kistemaker, H. A. V; Nardozza, A. P.; Overkleeft, H. S.; van der Marel, G. A.; Ladurner, A. G.; Filippov, D. V. *Angew. Chem., Int. Ed.* **2016**, *55*, 10634−10638.
(11) Fontana, P.; Bonfiglio, J. J.; Palazzo, L.; Bartlett, E.; Matic, I.; Ahel, I. *eLife* **2017**, *6*, e28533.
(12) Vivelo, C. A.; Leung, A. K. L. *Proteomics* **2015**, *15*, 203−217.
(13) Kistemaker, H. A. V; van der Heden van Noort, G. J.; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V. *Org. Lett.* **2013**, *15*, 2306−2309.
(14) Volbeda, A. G.; Kistemaker, H. A. V; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V.; Codée, J. D. C. *J. Org. Chem.* **2015**, *80*, 8796−8806.
(15) Bérces, A.; Whitfield, D. M.; Nukada, T.; do Santos Z, I.; Obuchowska, A.; Krepinsky, J. *Can. J. Chem.* **2004**, *82*, 1157−1171.
(16) Moss, J.; Tsai, S.; Adamik, R.; Chen, H.; Stanley, S. J. *Biochemistry* **1988**, *27*, 5819−5823.
(17) Moss, J.; Oppenheimer, N. J.; West, R. E.; Stanley, S. J. *Biochemistry* **1986**, *25*, 5408−5414.
(18) Palazzo, L.; Thomas, B.; Jemth, A.; Colby, T.; Leidecker, O.; Feijs, K. L. H.; Zaja, R.; Loseva, O.; Puigvert, J. C.; Matic, I.; Helleday, T.; Ahel, I. *Biochem. J.* **2015**, *468*, 293−301.
(19) Zhang, Y.; Wang, J.; Ding, M.; Yu, Y. *Nat. Methods* **2013**, *10*, 981−986.
(20) Wakabayashi, K.; Pigman, W. *Carbohydr. Res.* **1974**, *35*, 3−14.
(21) Goshe, M. B.; Conrads, T. P.; Panisko, E. A.; Angell, N. H.; Veenstra, T. D.; Smith, R. D. *Anal. Chem.* **2001**, *73*, 2578−2586.
(22) Oda, Y.; Nagasu, T.; Chait, B. T. *Nat. Biotechnol.* **2001**, *19*, 379−382.
(23) Wells, L.; Vosseller, K.; Cole, R. N.; Cronshaw, J. M.; Matunis, M. J.; Hart, G. W. *Mol. Cell. Proteomics* **2002**, *1*, 791−804.

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