Bioorthogonal Chemistry Hot Paper

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A Chemical Probe for Dehydrobutyrine

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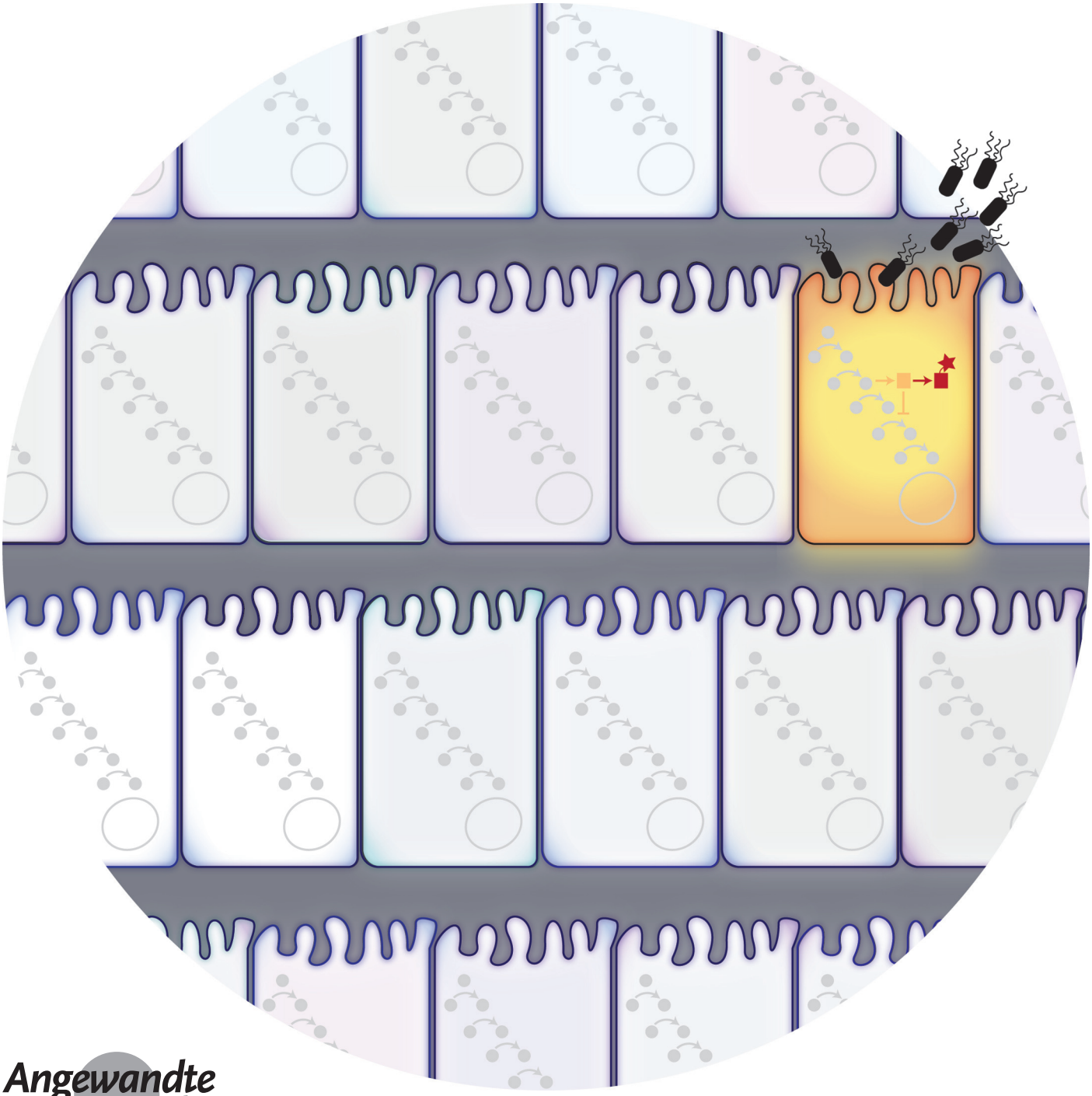

Abstract: *Bacterial phosphothreonine lyases, or phospholyases, catalyze a unique post-translational modification that introduces dehydrobutyrine (Dhb) or dehydroalanine (Dha) in place of phosphothreonine or phosphoserine residues, respectively. We report the use of a phospha-Michael reaction to label proteins and peptides modified with Dha or Dhb. We demonstrate that a nucleophilic phosphine probe is able to modify Dhb-containing proteins and peptides that were recalcitrant to reaction with thiol or amine nucleophiles under mild aqueous conditions. Furthermore, we used this reaction to detect multiple Dhb-modified proteins in mammalian cell lysates, including histone H3, a previously unknown target of phospholyases. This method should prove useful for identifying new phospholyase targets, profiling the biomarkers of bacterial infection, and developing enzyme-mediated strategies for bioorthogonal labeling in living cells.*

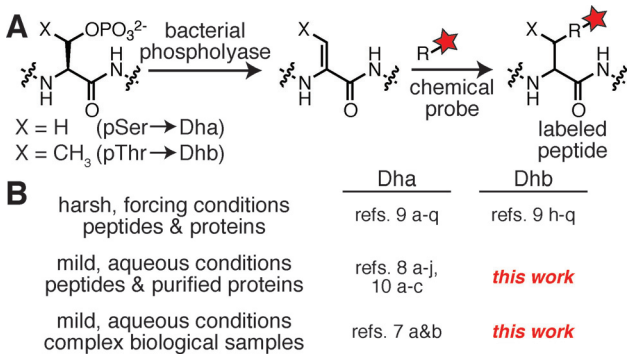

Figure 1. A) Bacterial phospholyases catalyze the elimination of phosphate from phosphoserine- or phosphothreonine-containing proteins to yield dehydroalanine (Dha) or dehydrobutyrine (Dhb), respectively. These electrophilic species can be captured using nucleophilic chemical probes. B) Extensive research has described the conjugate addition to Dha under a variety of conditions; however, Dhb has only been labeled using harsh conditions. This work establishes a method to label Dhb-bearing peptides and proteins under mild, biocompatible conditions in complex biological samples such as crude cellular lysates.

Bacterial phosphothreonine lyases, or phospholyases, are a family of virulence factors that have the unique ability to catalyze an irreversible β-elimination of phosphate from phosphorylated threonine (pThr) or serine (pSer) residues. This activity results in the formation of dehydrobutyrine (Dhb) or dehydroalanine (Dha), respectively, although pThr residues are the preferred phospholyase substrates (Figure 1 A).[1] Previous work has established that, during bacterial infection, phospholyases attenuate the innate immune response by deactivating mitogen-activated protein kinase (MAPK) signaling through β-elimination of phosphate from a key pThr within the ERK1/2 activation loop.[1b–f,2] Our recent work has demonstrated that phospholyases may have additional targets beyond the MAPK family.[1g] Identification of new phospholyase targets would lead to a better understanding of the infection process, and could uncover valuable biomarkers for bacterial infection. Since bacterial phospholyases prefer phosphothreonine substrates, profiling Dhb within the proteome is critical for the global analysis of phospholyase activity. However, the identification of phospholyase substrates remains challenging because there are currently no biocompatible methods for the selective enrichment of Dhb-modified proteins. Herein, we describe a nucleophilic phosphine that selectively labels Dhb-modified peptides and proteins under mild, biocompatible conditions (Figure 1 A). We further demonstrate that a biotinylated phosphine probe can be used to specifically label Dhb-containing proteins in the complex environment of mammalian cell lysate.

Electrophilic moieties are rare on proteins, thus making the α,β-unsaturated amide generated by phospholyases particularly interesting as a potential bioorthogonal chemical handle.[3] The Michael addition[4] of nucleophilic groups, especially thiols, to α,β-unsaturated functional groups is known to occur readily under biocompatible conditions.[5] Indeed, this reaction occurs naturally for lantibiotics[6] and has been used in cells to modify Dha-bearing proteins.[7] This approach has also been used to prepare mimics of post-translational modifications,[8] or, with more limited success, to enrich phosphoproteomic samples.[9] The majority of these reports focus on Dha, though many suggest that Dhb-containing proteins are amenable to modification using the same or similar conditions. However, only a few reports have described the use of a Michael addition to label proteins or peptides containing Dhb, all of which require high pH (10–13) and/or elevated temperatures (50–90 °C) to accelerate conjugate addition (Figure 1 B).[9h–q] Since phosphorylated and/or glycosylated Ser and Thr residues are susceptible to base-catalyzed elimination, such conditions would produce Dha and Dhb residues indistinguishable from those generated from genuine phospholyase activity. Therefore, there is a great need for reactions that can enable selective labeling of Dhb at near neutral pH using mild, aqueous conditions.

Our study was initiated by evaluating thiol-Michael addition chemistry for the modification of Dhb-bearing peptides and proteins compared to those bearing Dha. When Dha or Dhb-containing peptides (1 mM) derived from the activation loop of ERK1/2 [H-GFL-Dha-EYV-NH_2 (peptide **A**) or H-GFL-Dhb-EYV-NH_2 (peptide **B**)] were treated with 50 mM β-mercaptoethanol (βME) at 37 °C for one hour, peptide **A** was modified to 57 ± 1 %, whereas modification of peptide **B** was not detected. Even after 24 hours of incubation, only trace amounts of addition products to peptide **B** were observed, while peptide **A** was labeled quantitatively (Figure 2 A–C). Pseudo-first-order kinetic experiments were used to determine the second-order rate constant for the addition of βME to peptide **A** (k =

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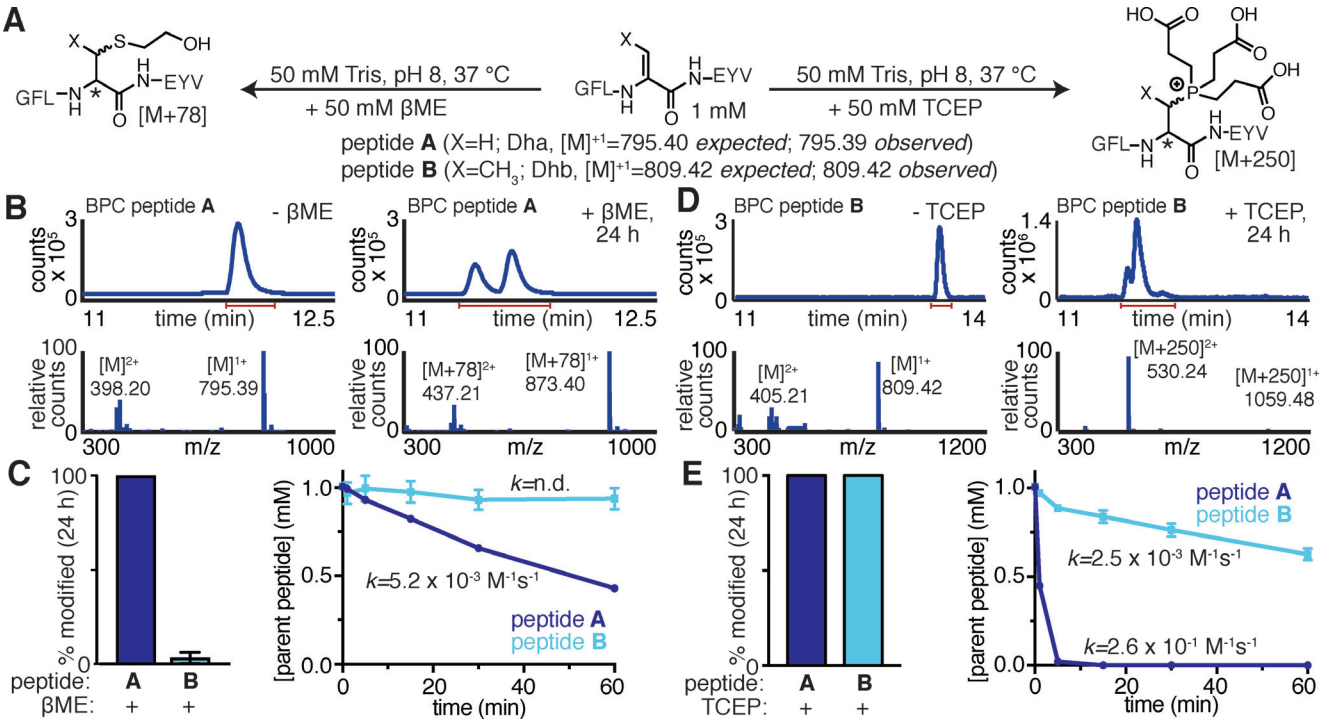

Figure 2. A) Reaction scheme for the conjugate addition of βME (left) or TCEP (right) to Dha- or Dhb-containing peptides (peptides **A** & **B**, respectively). B) After 24 h of treatment with βME, peptide **A** was quantitatively converted to the thioether product, as observed by LC–MS. Two distinct chromatographic peaks were observed, corresponding to the two expected diastereomeric products. C) Reactions that were incubated overnight resulted in quantitative conversion of peptide **A**, as measured by LC–MS. Rates of the thia-Michael addition of βME to peptides **A** and **B** were measured by loss of parent peptide as a function of time, as quantified by HPLC. D) After 24 h of incubation, both peptides **A** and **B** were modified by TCEP, as assessed by LC–MS. The four possible diastereomeric products could not be fully chromatographically resolved; however, it appears that there is a preference for one, as seen in the LC–MS chromatogram. E) After overnight reaction, quantitative conversion of both peptides **A** and **B** was achieved, as measured by LC–MS. Rates of phospha-Michael addition of TCEP to peptides **A** and **B** were measured by loss of parent peptide as a function of time, as quantified by HPLC.

$5.2 \times 10^{-3}\,\text{M}^{-1}\text{s}^{-1}$). However, the reaction was too slow to measure a rate constant for peptide **B** under the same conditions, thus further demonstrating that Dhb is resistant to addition by aliphatic thiols and is challenging to label under mild conditions. This behavior is likely due to the attenuated electrophilicity of Dhb relative to Dha that arises from the steric hindrance and hyperconjugation of the extra methyl group.

To determine whether other nucleophiles could enable Dhb modification, we evaluated the reaction between a panel of nucleophiles and peptide **B**. Although a variety of thiols and amines were able to modify Dhb under forcing conditions, we found that this was not the case when employing more mild conditions (Table S1 and Figure S1 in the Supporting Information). When using biocompatible conditions, all of the thiol-containing compounds that were tested yielded extensive modification of peptide **A** after 24 hours but resulted in only minimal modification of peptide **B** (0–11.5% modified). The aza-Michael reaction was recently reported as a robust method for the modification of Dha-bearing proteins.[5g,8a–c,9f–j] A variety of primary and secondary amines were tested, but none could label peptide **B** under more stringent, buffered conditions. Furthermore, none were more effective than thiol nucleophiles with respect to the modification of peptide **A** (Table S1 and Figure S1).

Based on these findings, it became apparent that the modification of Dhb-containing proteins and peptides presents a chemical challenge compared to the labeling of those bearing Dha, especially under mild aqueous conditions. Thus, we turned to a few recent studies that have described new reactions to modify α,β-unsaturated functional groups (Figure 2, Figure S2).[10,11] In particular, a phospha-Michael addition was recently reported to occur between tris(2-carboxyethyl)phosphine (TCEP) and proteins bearing α,β-unsaturated amides (Figure 2A).[11] When peptides **A** or **B** (1 mM) were treated with TCEP (50 mM) at pH 8 and 37 °C, we found that both were modified after just one hour of incubation (100% and 37 ± 3% modified, respectively). Quantitative modification was achieved after 24 hours of incubation, as assessed by LC–MS (Figure 2D,E). We found that the extent of modification of peptide **B** was within roughly 15% of that observed for a Dhb-bearing polyalanine peptide of the same length, thus indicating that the peptide sequence can have a minor influence but does not dramatically impact the reaction success (Figure S3). To assess the stability of the β-phosphonium product, LC–MS was used to monitor a sample of purified TCEP-modified peptide **B**, which exhibited no degradation after four weeks of incubation at 37 °C (Figure S4).

We determined the second-order rate constant for the phosphine addition to peptide **A** ($k = 2.6 \times 10^{-1}\,\text{M}^{-1}\text{s}^{-1}$) and found it to be roughly two orders of magnitude faster than the reaction with the thiol ($k = 5.2 \times 10^{-3}\,\text{M}^{-1}\text{s}^{-1}$) under the same conditions. These values indicate that the phospha-Michael reaction is kinetically favored, which is in agreement with previous reports.[11a] Phosphines are well-known to have enhanced nucleophilicity relative to thiols, and are frequently used as catalysts, notably in Michael and Bayliss-Hillman reactions.[12] In the case of TCEP, prior work has demonstrated that its carboxylic acid groups are critical for the reaction, likely due to internal charge stabilization of the β-phosphonium product.[11b] Moreover, the TCEP phosphine has a lowered pK_a (p$K_a = 7.6$) compared to that of the βME thiol (p$K_a = 9.6$), and is therefore better primed for reaction at near-neutral pH.[11a,13] Owing to the enhanced kinetics of the phospha-Michael reaction, it was possible to determine the second-order rate constant for Dhb-containing substrates ($k = 2.5 \times 10^{-3}\,\text{M}^{-1}\text{s}^{-1}$), which was similar to that observed for crotonyl substrates ($k = 6 \times 10^{-4}\,\text{M}^{-1}\text{s}^{-1}$).[11b] Although the reaction with Dha is just over 100-fold faster than the reaction with Dhb, the phospha-Michael reaction was remarkably faster than the thia- or aza-mediated versions for the labeling of Dhb. Thus, this is the first reaction reported to date that is capable of modifying Dhb-bearing species under biocompatible conditions.

To leverage this new reaction in a cellular environment, we prepared biotinylated probes that could detect Dhb-bearing cellular proteins following exposure to phospholyases (Figure 3A). We synthesized a biotin-containing thiol probe (**1**) as well as a biotin-containing phosphine probe (**2**; Figure 3B).[11b] For these studies, we cultured A431 cells, which overexpress epidermal growth factor (EGF) receptors and exhibit constitutive activation of downstream kinases, including ERK1/2.[14] When A431 cell lysates were treated with recombinant OspF, a phospholyase from *Shigella flexneri*, we observed complete loss of the signal corresponding to phosphorylated ERK1/2, although total ERK1/2 levels were unchanged (Figure 3C). This is consistent with efficient β-elimination of phosphate from pThr in the ERK1/2 activation loop (-GFLpTEpYV-).[1] Following OspF treatment, lysates were exposed to either probe **1** or probe **2** (1 mM); a biotinylated band appeared at the exact molecular weight expected for ERK1/2 when lysates were treated with probe **2**, but not with **1** (Figure 3C, red arrow; Figures S5,S6). Subsequent immunoprecipitation of biotinylated species from A431 lysates treated with probe **2** confirmed that ERK1/2 is a cellular target of OspF (Figure S7). These results demonstrate that probe **2** can successfully modify Dhb-containing proteins in cell lysates, while probe **1** cannot.

Furthermore, non-specific labeling of cellular proteins was substantially greater for probe **1** than for probe **2**, thus suggesting that the phosphine probe is more selective for its intended cellular targets (Figure 3C & S6). To date, Dhb-modified proteins have not been reported to occur natively in mammalian cells. However, probe **2** does have the potential to modify endogenous α,β-unsaturated electrophiles such as fumarate and/or crotonylate. Indeed, past efforts have used probe **2** to detect protein lysine crotonylation. However, those

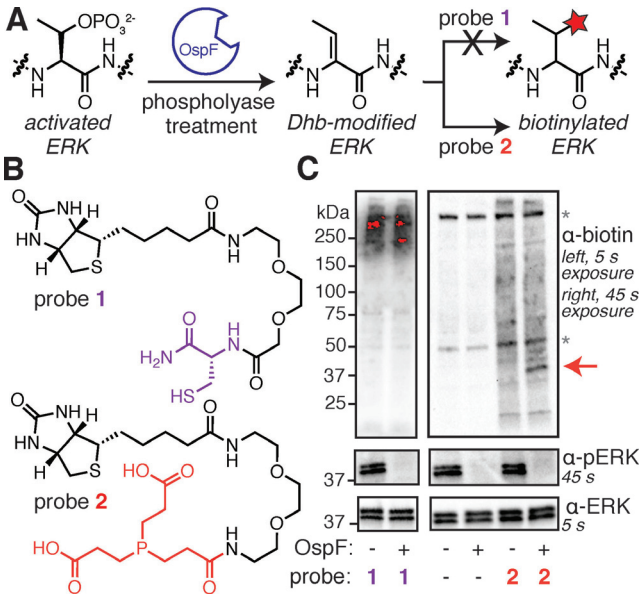

Figure 3. A) Phosphorylated cellular targets, such as activated ERK1/2, can be eliminated upon treatment with OspF, a phospholyase. The resulting Dhb-modified protein(s) can be subsequently labeled upon treatment with a nucleophilic probe. B) Chemical structures of biotinylated probes **1** and **2**. C) A431 cell lysates were treated ± OspF (50 nM) and ± probe **1** or **2** (1 mM). Phosphorylated ERK1/2 is eliminated upon treatment with OspF; the resulting Dhb-modified ERK can be further biotinylated by probe **2**, but not **1**, as assessed by western blot (red arrow). The level of non-specific background was substantially greater for probe **1**, which necessitated a shorter exposure time during western blot development (5 s vs. 45 s for probe **2**; see also Figure S6). Non-specific bands that appeared upon treatment with α-biotin antibodies independent of probe addition are indicated by (*).

studies utilized substantially higher probe concentrations (4 mM) and required additional steps to isolate histone fractions or nuclear extracts prior to probe exposure. To assess whether protein crotonylation could account for the background signal observed in the absence of OspF, we probed for histone H3, a well-known target of lysine crotonylation,[11b] following immunoprecipitation of biotinylated species from cell lysates treated with probe **2** (Figure S7). We found no evidence of H3 pulldown in the absence of OspF, thus suggesting that endogenous background from crotonylation is minimal under the conditions used in this study. However, a strong signal was observed for H3 pulldown in lanes that were treated with both OspF and probe **2**. Past studies have demonstrated that OspF can localize in the nucleus and have proposed that OspF inhibits H3 phosphorylation through an indirect mechanism.[15] In contrast, our results indicate that phosphorylated histone H3 is a direct target for OspF and that the resulting Dhb-modified H3 is effectively labeled by probe **2**. Thus, this work demonstrates that probe **2** can be used for the detection of multiple Dhb-modified proteins in crude mammalian cell lysates.

Currently, the only confirmed mammalian targets of bacterial phospholyases are MAP kinases, including ERK1/2. Our prior work implied that phospholyases may have additional targets outside of the MAPK family.[1g] Moreover,

this work has serendipitously identified histone H3 as a previously undiscovered target of OspF (Figure S7). To further explore this hypothesis, we treated A431 cell lysates with increasing concentrations of OspF and labeled the proteome with probe **2**. This revealed several biotinylated bands at molecular weights higher than expected for MAPKs (Figure S8). Building on these findings, our future work will use this method to capture Dhb-modified proteins from cellular samples, thereby enabling the identification of new phospholyase targets. The discovery of such targets is an essential step towards elucidating the complete role of phospholyases during bacterial infection, and for the identification of new biomarkers of exposure to pathogens including *Shigella flexneri* or *Salmonella enterica* Typhimurium. Moreover, the method developed herein will likely be useful in a range of bioconjugation applications. In particular, although the reduced electrophilicity of Dhb relative to Dha renders it less reactive, it also is recalcitrant to modification by cellular thiols and amines. This behavior renders the phospha-Michael reaction with Dhb much more selective than the same reaction with Dha. As a result, the phospha-Michael reaction with Dhb could be particularly advantageous for the development of bioorthogonal labeling strategies that can be employed in living cells, thus making it an invaluable addition to the bioconjugation toolkit.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioconjugation · bioorthogonal chemistry · lyases · Michael addition · protein modification

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Angew. Chem. **2020**, *132*, 7420–7425

[1] a) D. F. Brennan, D. Barford, *Trends Biochem. Sci.* **2009**, *34*, 108–114; b) H. Li, H. Xu, Y. Zhou, J. Zhang, C. Long, S. Li, S. Chen, J.-M. Zhou, F. Shao, *Science* **2007**, *315*, 1000–1003; c) P. Mazurkiewicz, J. Thomas, J. A. Thompson, M. Liu, L. Arbibe, P. Sansonetti, D. W. Holden, *Mol. Microbiol.* **2008**, *67*, 1371–1383; d) Y. Zhu, H. Li, C. Long, L. Hu, H. Xu, L. Liu, S. Chen, D. C. Wang, F. Shao, *Mol. Cell* **2007**, *28*, 899–913; e) Z. Ke, G. K. Smith, Y. Zhang, H. Guo, *J. Am. Chem. Soc.* **2011**, *133*, 11103–11105; f) L. Chen, H. Wang, J. Zhang, L. Gu, N. Huang, J. M. Zhou, J. Chai, *Nat. Struct. Mol. Biol.* **2008**, *15*, 101–102; g) K. A. Chambers, N. S. Abularrage, R. A. Scheck, *Biochemistry* **2018**, *57*, 3790–3796.

[2] J. Zhang, F. Shao, Y. Li, H. Cui, L. Chen, H. Li, Y. Zou, C. Long, L. Lan, J. Chai, S. Chen, X. Tang, J. M. Zhou, *Cell Host Microbe* **2007**, *1*, 175–185.

[3] a) G. L. Holliday, D. E. Almonacid, J. B. O. Mitchell, J. M. Thornton, *J. Mol. Biol.* **2007**, *372*, 1261–1277; b) M. L. Matthews, L. He, B. D. Horning, E. J. Olson, B. E. Correia, J. R. Yates, P. E. Dawson, B. F. Cravatt, *Nat. Chem.* **2017**, *9*, 234–243.

[4] A. Michael, *Am. Chem. J.* **1887**, *9*, 112–124.

[5] a) H. M. Seifert, K. R. Trejo, E. V. Anslyn, *J. Am. Chem. Soc.* **2016**, *138*, 10916–10924; b) J. M. Bradshaw, J. M. McFarland, V. O. Paavilainen, A. Bisconte, D. Tam, V. T. Phan, S. Romanov, D. Finkle, J. Shu, V. Patel, T. Ton, X. Y. Li, D. G. Loughhead, P. A. Nunn, D. E. Karr, M. E. Gerritsen, J. O. Funk, T. D. Owens, E. Verner, K. A. Brameld, R. J. Hill, D. M. Goldstein, J. Taunton, *Nat. Chem. Biol.* **2015**, *11*, 525–531; c) S. Krishnan, R. M. Miller, B. X. Tian, R. D. Mullins, M. P. Jacobson, J. Taunton, *J. Am. Chem. Soc.* **2014**, *136*, 12624–12630; d) I. M. Serafimova, M. A. Pufall, S. Krishnan, K. Duda, M. S. Cohen, R. L. Maglathlin, J. M. McFarland, R. M. Miller, M. Frodin, J. Taunton, *Nat. Chem. Biol.* **2012**, *8*, 471–476; e) E. Weerapana, G. M. Simon, B. F. Cravatt, *Nat. Chem. Biol.* **2008**, *4*, 405–407; f) G. T. Hermanson, *Bioconjugate Techniques*, 3rd ed., Academic Press, New York, **2013**, pp. 1–1146; g) B. N. Naidu, M. E. Sorenson, T. P. Connolly, Y. Ueda, *J. Org. Chem.* **2003**, *68*, 10098–10102.

[6] a) M. A. Ortega, W. A. van der Donk, *Cell Chem. Biol.* **2016**, *23*, 31–44; b) L. M. Repka, J. R. Chekan, S. K. Nair, W. A. van der Donka, *Chem. Rev.* **2017**, *117*, 5457–5520.

[7] a) M. P. C. Mulder, K. Witting, I. Berlin, J. N. Pruneda, K. P. Wu, J. G. Chang, R. Merkx, J. Bialas, M. Groettrup, A. C. O. Vertegaal, B. A. Schulman, D. Komander, J. Neefjes, F. El Oualid, H. Ovaa, *Nat. Chem. Biol.* **2016**, *12*, 523–530; b) R. Meledin, S. M. Mali, O. Kleifeld, A. Brik, *Angew. Chem. Int. Ed.* **2018**, *57*, 5645–5649; *Angew. Chem.* **2018**, *130*, 5747–5751.

[8] a) P. M. T. Ferreira, H. L. S. Maia, L. S. Monteiro, J. Sacramento, J. Sebastiao, *J. Chem. Soc. Perkin Trans. 1* **2000**, 3317–3324; b) J. Dadová, K. J. Wu, P. G. Isenegger, J. C. Errey, G. J. L. Bernardes, J. M. Chalker, L. Raich, C. Rovira, B. G. Davis, *ACS Cent. Sci.* **2017**, *3*, 1168–1173; c) A. M. Freedy, M. J. Matos, O. Boutureira, F. Corzana, A. Guerreiro, P. Akkapeddi, V. J. Somovilla, T. Rodrigues, K. Nicholls, B. Xie, G. Jimenez-Oses, K. M. Brindle, A. A. Neves, G. J. L. Bernardes, *J. Am. Chem. Soc.* **2017**, *139*, 18365–18375; d) G. J. L. Bernardes, J. M. Chalker, J. C. Errey, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 5052–5053; e) J. M. Chalker, L. Lercher, N. R. Rose, C. J. Schofield, B. G. Davis, *Angew. Chem. Int. Ed.* **2012**, *51*, 1835–1839; *Angew. Chem.* **2012**, *124*, 1871–1875; f) K. P. Chooi, S. R. G. Galan, R. Raj, J. McCullagh, S. Mohammed, L. H. Jones, B. G. Davis, *J. Am. Chem. Soc.* **2014**, *136*, 1698–1701; g) J. T. Guo, J. Y. Wang, J. S. Lee, P. G. Schultz, *Angew. Chem. Int. Ed.* **2008**, *47*, 6399–6401; *Angew. Chem.* **2008**, *120*, 6499–6501; h) M. R. J. Vallée, M. W. Schombs, Z. J. Balaban, J. Colyer, B. G. Davis, *Chem. Commun.* **2016**, *52*, 3014–3017; i) J. Wang, S. M. Schiller, P. G. Schultz, *Angew. Chem. Int. Ed.* **2007**, *46*, 6849–6851; *Angew. Chem.* **2007**, *119*, 6973–6975; j) S. R. G. Galan, J. R. Wickens, J. Dadova, W. L. Ng, X. L. Zhang, R. A. Simion, R. Quinlan, E. Pires, R. S. Paton, S. Caddick, V. Chudasama, B. G. Davis, *Nat. Chem. Biol.* **2018**, *14*, 955–963.

[9] a) G. Arrigoni, S. Resjo, F. Levander, R. Nilsson, E. Degerman, M. Quadroni, L. A. Pinna, P. James, *Proteomics* **2006**, *6*, 757–766; b) M. B. Goshe, T. P. Conrads, E. A. Panisko, N. H. Angell, T. D. Veenstra, R. D. Smith, *Anal. Chem.* **2001**, *73*, 2578–2586; c) Y. Oda, T. Nagasu, B. T. Chait, *Nat. Biotechnol.* **2001**, *19*, 379–382; d) A. J. Thompson, S. R. Hart, C. Franz, K. Barnouin, A. Ridley, R. Cramer, *Anal. Chem.* **2003**, *75*, 3232–3243; e) K.

Vosseller, K. C. Hansen, R. J. Chalkley, J. C. Trinidad, L. Wells, G. W. Hart, A. L. Burlingame, *Proteomics* **2005**, *5*, 388 – 398; f) W. D. Annan, W. Manson, J. A. Nimmo, *Anal. Biochem.* **1982**, *121*, 62 – 68; g) T. Mega, Y. Hamazume, Y. M. Nong, T. Ikenaka, *J. Biochem.* **1986**, *100*, 1109 – 1116; h) L. Wells, K. Vosseller, R. N. Cole, J. M. Cronshaw, M. J. Matunis, G. W. Hart, *Mol. Cell. Proteomics* **2002**, *1*, 791 – 804; i) T. Mega, N. Nakamura, T. Ikenaka, *J. Biochem.* **1990**, *107*, 68 – 72; j) K. Mattila, J. Siltainsuu, L. Balaspiri, M. Ora, H. Lonnberg, *Org. Biomol. Chem.* **2005**, *3*, 3039 – 3044; k) E. Salih, W. L. Siqueira, E. J. Helmerhorst, F. G. Oppenheim, *Anal. Biochem.* **2010**, *407*, 19 – 33; l) H. C. Tseng, H. Ovaa, N. J. Wei, H. Ploegh, L. H. Tsai, *Chem. Biol.* **2005**, *12*, 769 – 777; m) H. Nika, E. Nieves, D. H. Hawke, R. H. Angeletti, *J. Biomol. Tech.* **2013**, *24*, 132 – 153; n) H. Jaffe, Veeranna, H. C. Pant, *Biochemistry* **1998**, *37*, 16211 – 16224; o) M. P. Molloy, P. C. Andrews, *Anal. Chem.* **2001**, *73*, 5387 – 5394; p) W. Li, R. A. Boykins, P. S. Backlund, G. Y. Wang, H. C. Chen, *Anal. Chem.* **2002**, *74*, 5701 – 5710; q) Z. A. Knight, B. Schilling, R. H. Row, D. M. Kenski, B. W. Gibson, K. M. Shokat, *Nat. Biotechnol.* **2003**, *21*, 1047 – 1054.

[10] a) T. H. Wright, B. J. Bower, J. M. Chalker, G. J. L. Bernardes, R. Wiewiora, W.-L. Ng, R. Raj, S. Faulkner, M. R. J. Vallée, A. Phanumartwiwath, O. D. Coleman, M.-L. Thézénas, M. Khan, S. R. G. Galan, L. Lercher, M. W. Schombs, S. Gerstberger, M. E. Palm-Espling, A. J. Baldwin, B. M. Kessler, T. D. W. Claridge, S. Mohammed, B. G. Davis, *Science* **2016**, *6312*, aag1465; b) T. H. Wright, B. G. Davis, *Nat. Protoc.* **2017**, *12*, 2243 – 2250; c) A. Yang, S. Ha, J. Ahn, R. Kim, S. Kim, Y. Lee, J. Kim, D. Soll, H. Y. Lee, H. S. Park, *Science* **2016**, *354*, 623 – 626.

[11] a) Y. J. Lee, Y. Kurra, W. R. Liu, *ChemBioChem* **2016**, *17*, 456 – 461; b) J. Bos, T. W. Muir, *J. Am. Chem. Soc.* **2018**, *140*, 4757 – 4760.

[12] a) J. L. Methot, W. R. Roush, *Adv. Synth. Catal.* **2004**, *346*, 1035 – 1050; b) H. C. Guo, Y. C. Fan, Z. H. Sun, Y. Wu, O. Kwon, *Chem. Rev.* **2018**, *118*, 10049 – 10293.

[13] W. A. Henderson, S. A. Buckler, *J. Am. Chem. Soc.* **1960**, *82*, 5794 – 5800.

[14] S. Boldt, U. H. Weidle, W. Kolch, *Carcinogenesis* **2002**, *23*, 1831 – 1838.

[15] a) D. V. Zurawski, C. Mitsuhata, K. L. Mumy, B. A. McCormick, A. T. Maurelli, *Infect. Immun.* **2006**, *74*, 5964 – 5976; b) H. Harouz, C. Rachez, B. M. Meijer, B. Marteyn, F. Donnadieu, F. Cammas, C. Muchardt, P. Sansonetti, L. Arbibe, *EMBO J.* **2014**, *33*, 2606 – 2622.

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