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Irreversible Protein Labeling by Paal–Knorr Conjugation

Ramesh Dasari[a], **Dr. James J. La Clair**[b],[c], and **Prof. Alexander Kornienko**[a]

[a]Department of Chemistry and Biochemistry, Texas State University San Marcos, TX 78666 (USA)

[b]Department of Chemistry and Biochemistry University of California at San Diego La Jolla, CA 92093 (USA)

[c]Xenobe Research Institute P. O. Box 3052, San Diego, CA 92163 (USA)

Abstract

The application of new chemical reactions in a biological context has advanced bioconjugation methods for both fundamental research and commercial arenas. Recent adaptations of reactions such as Huisgen 1,3-dipolar or Diels–Alder cycloadditions have enabled the labeling of specific residues in biomolecules by the attachment of molecules carrying azides, alkynes, or strained alkenes. Although these are fundamental tools, there is a need for the discovery of reactions that can label native proteins. We report herein the adaptation of the Paal–Knorr reaction to label lysine residues in proteins via pyrrole linkages.

Keywords

biocompatible reactions; bioconjugation; click chemistry; Paal–Knorr reaction; protein labeling

New protein-labeling methods, characterized by efficiency, short reaction times, site-selective targeting, or the use of low substrate concentrations, are in great demand.[1] Many reported labeling reagents for native proteins[2] target strongly nucleophilic cysteine residues.[3] Covalent modification of lysine (Lys) is another important strategy, although the nucleophilicity of Lys-NH_2 is diminished by its protonated nature under physiological conditions (pK_a = 10.5). Some recently reported Lys-targeting methods include reductive alkylation using iridium-catalyzed transfer hydrogenation,[4] coupling with diazonium terephthalates,[5] Michael addition with allylic elimination of sulfonamides,[6] sulfonylation with fluorosulfonylbenzoates,[7] and nucleophilic aromatic substitution with dichlorotriazines.[8] We describe here the advancement of a class of labels (Scheme 1) that target Lys residues by the formation of irreversible pyrrole linkages.

One of the oldest named reactions involving primary amines is the classical Paal–Knorr pyrrole synthesis that was reported independently by Paal[9] and Knorr[10] in 1884. The

Correspondence to: James J. La Clair; Alexander Kornienko.

Conflict of Interest

The authors declare no conflict of interest.

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reaction, as depicted in Scheme 2, involves the condensation of a primary amine with 1,4-dicarbonyl compound **3** in the presence of an acid catalyst to give pyrroles **5** through the intermediary hemiaminal **4**.[11]

As the Paal–Knorr reaction proceeds under mild reaction conditions, often at room temperature with the use of a mild acid, it is a method of choice for sensitive functionality. This has been well documented through a variety of synthetic applications, such as the commercial preparation of the anti-cholesterolemic drug atorvastatin (Lipitor)[12] or total synthesis of natural products, such as that of roseophilin (reported by Trost[13]).

The Paal–Knorr reaction is also biologically relevant, based on its involvement in *n*-hexane-induced axonal atrophy within the central nervous system. As shown in both in vitro and in vivo systems, hexane-2,5-dione (R^1 =R^2=Me, Scheme 2), a neurotoxic metabolite of *n*-hexane, undergoes a selective Paal–Knorr reaction with lysine residues (R^3NH_2, Scheme 2) of axonal cytoskeleton proteins, forming 2,5-dimethylpyrrole adducts within specific regions of neurofilaments.[14] Based on this biological precedent, it appeared to us that the Paal–Knorr reaction would be an interesting addition to the arsenal of lysine-labeling methods. We focused our efforts on demonstrating this method through probe **1**, as shown in Scheme 3.

The synthesis of Paal–Knorr probes **1** and **2** began with the Wittig reaction of 5-methylfurfural with Ph_3P = $CHCO_2Me$ to afford the corresponding α,β-unsaturated ester, which was hydrogenated to yield furan ester **9** (Scheme 4). Ester **9** was subjected to an acid-catalyzed hydrolytic opening of the furan ring with concomitant ester hydrolysis to afford 4,7-dioxooctanoic acid (**10**) in 62 % yield (Scheme 4). The resulting acid was coupled with immunoaffinity fluorescent (IAF) tag **12**[15] using EDAC and DMAP to afford the desired probe **1** in 86 % yield. The absence of any detectable aldol processes under both acidic (**9** to **10**) and basic (**10** to **1**) reaction conditions attests to the stability of such aliphatic 1,4-dicarbonyl compounds and bodes well for their applications in a biological context.

In contrast, we found that probe **1** was highly reactive toward primary amines, as was evident by the facile condensation with benzyl amine to form pyrrole **13** in an excellent 86 % yield under mildly acidic conditions at 23 °C (Scheme 4). Similar methods were then applied to prepare chain-elongated probe **2** from **10** (see the Supporting Information). This probe was prepared to further explore the linker requirements between the IAF tag and the reactive moiety. In addition, we also synthesized monoketone **14** (Scheme 5, see the Supporting Information) as a control. Here, the presence of a single ketone in **14** would prevent it from undergoing the Paal–Knorr reaction and instead lead to Schiff base formation (Scheme 5).

We then examined the use of probes **1** and **2** to modify two proteins (Scheme 3), bovine serum albumin (BSA) and keyhole limpet serum (KLH), commonly conjugated through their lysine residues for antibody production.[16] As shown in Figure 1a, probes **1** and **2** were able to label BSA and KLH. The fact that both probes operated at similar efficacies (Figure 1a) indicated that both short linkers (**1**) and longer linkers (**2**) were viable. For both proteins, the reaction was strongest with probes **1** and **2**, although a low level of labeling was

observed with the monoketone control (**14**; Figure 1a). After further analysis, we found that this background labeling of BSA with **14** could be removed by spin dialysis prior to gel analysis; however, traces of fluorescence from **14**, likely resulting from Schiff base formation, were still observed with KLH. Comparative analyses[17] of the levels of fluorescence in Figure 1a with standards indicated that 1.4 ± 0.1 and 2.4 ± 0.2 tags were observed per protein for BSA and KLH, respectively, after treatment with probe (50 equiv) in phosphate-buffered saline (PBS) at pH 7.2 for 24 h at 37 °C.

Experiments with other proteins revealed that the Paal–Knorr conjugation was not unique to BSA and KLH. Of interest, labeling of LC-8, a protein known to oligomerize,[19] was accompanied by increased levels of dimeric and trimeric oligomers (Figure 1c). These oligomers did not revert upon addition of a reducing agent (BME, DTT, or TCEP), boiling the sample prior to SDS-PAGE analysis, or changing the type of gel; this indicated that they might have arisen from covalent modifications, possibly involving crosslinking of the pyrrole units[20] brought into proximity by LC-8 oligomerizaton. While attempts were made to identify this crosslinking by mass spectral analyses, the complexity of finding trace levels of crosslinked peptides in this sample proved too difficult. This result provides early evidence that the Paal–Knorr technique may also be used to trap oligomerization states within proteins or even act on protein complexes.

We next tested the limits of the method. We found that visualization by eye was limited by treatment with a tenfold excess of probe **1** when loading 1 μg of protein per lane on a typical SDS-PAGE gel (Figure 1b). Using a Typhoon scanner, the labeling of BSA could be visualized after treatments with 0.5–2.5 equivalents of probe **1** (Figure 2a). For both, visualization improved after fixation with 10 % AcOH in 30 % aq. EtOH, as this removed background resulting from unreacted **1**. The detection limit of the labeling process was further reduced by Western blot analyses using an anti-IAF monoclonal antibody (mAb).[18] As illustrated in Figure 2b, we were able to detect labeling in bands containing >1 ng of BSA treated with 5 equivalents of **1**.

We also conducted studies to identify the optimal labeling conditions for BSA. We found that the reaction could be observed within 1 h at 37 °C, and labeling was not enhanced with longer treatment times, as indicated by SDS-PAGE analysis (Figure 1b). Thus, we typically allowed the reaction to run for 6 to 12 h to ensure completion of the pyrrole formation (Scheme 2). We also observed that reducing the pH did not influence the outcome of this reaction. Although pH 4–6 is often used to accelerate Paal–Knorr reactions, a reduction in pH did not seem to affect the ability of **1** to label BSA (Figure 2c). Finally, we carefully checked the spectroscopic properties of gel-purified **1**·BSA conjugate and found that its spectral properties matched that of **1** (Figure 2d).

Next, we set out to confirm that protein labeling of BSA occurred through the pyrrole-forming reaction, as illustrated in Scheme 3. Using 1 and 24 h treatments at 37 °C, we conducted labeling on 100 μg scale using 10 equivalents of **1**. Once complete, we purified the resulting protein by SDS-PAGE. Samples of the bands containing **1**-labeled BSA were collected under clean conditions and dried. These samples were submitted to trypsin digestion and LC-MS/MS, returning excellent peptide coverage with multiple long peptides

(Figure 3b).[22] We then conducted a detailed search of the identified peptides with MS-GF +[23] and MODA[24] database search algorithms that enable the identification of peptides with non-natural modifications. Within this dataset, modified peptides were identified, such as S^{310}HCIAEVEKDAIPENLPPLTADFAE-D**K**^{336}DV^{338} (orange, Figure 3). Samples treated for 1 h contained peaks for both hemiaminal **4** (3567.6937 *m*/*z* obs., 3567.4706 *m*/*z* calcd) and pyrrole adduct **5** (*M*+407, 3532.6377 *m*/*z* obs., 3531.8295 *m*/*z* calcd). However, the sample treated for 24 h only contained the peak due to pyrrole **5**. Although this peptide could be detected, the low level and complexity of detection did not allow for an analytical evaluation of the site selectivity within this reaction.

We then evaluated the stability of these conjugates. Samples of **1**-labeled BSA or **1**-labeled KLH remained fluorescent and intact at pH 4–9. Moreover, the tagged protein did not degrade after long-term storage, as samples of **1**-labeled BSA used in Figure 3 were stored at 4°C for more than one month (limit of testing) or in lyophilized dry form at room temperature for more than one month (limit of testing), as evaluated by SDS-PAGE.

With stable materials in hand, we evaluated whether our conjugates were viable for cellular work. Using confocal microscopy, we observed rapid endocytosis and localization of **1**-labeled BSA within viable HCT-116 colon cancer cells (Figure 4). Subsequent staining with LysoTracker Red DND-99,[25] a red fluorescent lysosomal organelle stain, confirmed that **1**-labeled BSA was observed in the lysosomes as previously reported for BSA conjugates in HCT-116 cells.[26] Using western blot analyses (Figure S1 in the Supporting Information), we were able to confirm the fact that **1**-labeled BSA remained intact within the cells during this process.

In conclusion, we have demonstrated the application of the Paal–Knorr reaction for protein labeling using lysine-rich proteins as substrates (BSA, KLH, or LC-8). There are several features of this reaction that make its use for protein conjugation applications and chemical biological studies attractive. First, there are no reagents required or special media. In these examples, labeling was readily observed at pH 7.2 in PBS at ambient temperature. The labeling process occurred rapidly and was completed within 24 h. Second, while we demonstrated the labeling application using an IAF tag, the ease in synthesis of the warhead unit **10** (Scheme 4) allows tags to be appended to a wide range of agents. Third, probes containing warhead **10** are not reactive to media like many of the other functional handles, such as acrylates, α-haloamides, succinimidyl esters, or sulfonyl halides; an issue that often creates significant batch-based inhomogeneity, which has recently been addressed in part with continuous-flow reactors.[27] Finally, the fact that 1,4-diketones are resistant to metabolism[28] and have demonstrated Paal–Knorr reactivity in humans[14] indicate their potential for further advance as next generation tools for chemical biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Boutureira O, Bernardes GJL. Chem Rev. 2015; 115:2174. [PubMed: 25700113]
2. MacDonald JI, Munch HK, Moore T, Francis MB. Nat Chem Biol. 2015; 11:326. [PubMed: 25822913]
3. Chalker JM, Bernardes GJL, Lin YA, Davis B. Chem Asian J. 2009; 4:630. [PubMed: 19235822]
4. McFarland JM, Francis MB. J Am Chem Soc. 2005; 127:13490. [PubMed: 16190700]
5. Diethelm S, Schafroth MA, Carreira EM. Org Lett. 2014; 16:3908. [PubMed: 25019948]
6. Jinno F, Yoneyama T, Morohashi A, Kondo T, Asahi S. Biopharm Drug Dispos. 2011; 32:408. [PubMed: 21887787]
7. Gushwa NN, Kang S, Chen J, Taunton J. J Am Chem Soc. 2012; 134:20214. [PubMed: 23190395]
8. Shannon DA, Banerjee R, Webster ER, Bak DW, Wang C, Weerapana E. J Am Chem Soc. 2014; 136:3330. [PubMed: 24548313]
9. Paal C. Chem Ber. 1884; 17:2756.
10. Knorr L. Chem Ber. 1884; 17:1635.
11. Abbat S, Dhaked D, Arfeen M, Bharatam PV. RSC Adv. 2015; 5:88353.
12. Novozhilov YV, Dorogov MV, Blumina MV, Smirnov AV, Krasavin M. Chem Cent J. 2015; 9:7. [PubMed: 25705254]
13. Trost B, Doherty GA. J Am Chem Soc. 2000; 122:3801.
14. Zhang L, Gavin T, DeCaprio AP, LoPachin RM. Toxicol Sci. 2010; 117:180. [PubMed: 20554699]
15. Alexander MD, Burkart MD, Leonard MS, Portonovo P, Liang B, Ding X, Joullié MM, Gulledge BM, Aggen JB, Chamberlin AR, Sandler J, Fenical W, Cui J, Gharpure SJ, Polosukhin A, Zhang HR, Evans PA, Richardson AD, Harper MK, Ireland CM, Vong BG, Brady TP, Theodorakis EA, La Clair JJ. ChemBioChem. 2006; 7:409. [PubMed: 16432909]
16. Coligan JE, Tam JP, Shao J. Curr Protoc Neurosci. 2001; 5(Unit 5.6)
17. Fluorescence labeling was determined in gel by using densitometry measurements and parallel fluorescence with detection and SilverQuest stained gels using ImageJ, see: Rehbein P, Schwalbe H. Protein Expression Purif. 2015; 110:1.Rueden CT, Eliceiri KW. BioTechniques. 2007; 43:S31.
18. Yu WL, Guizzunti G, Foley TL, Burkart MD, La Clair JJ. J Nat Prod. 2010; 73:1659. [PubMed: 20836515]
19. a) Hall J, Hall A, Pursifull N, Barbar E. Biochemistry. 2008; 47:11940. [PubMed: 18942858] b) Barbar E. Biochemistry. 2008; 47:503. [PubMed: 18092820]
20. Pyrrole polymerization can occur in oxidative or low pH environments: Smith GF. Adv Heterocycl Chem. 1963; 2:287.Tan Y, Ghandi K. Synt Metals. 2013; 175:183.. Its occurrence within proteins might have some utility for site-directed polymerization of proteins: Albayrak C, Schwartz JR. ACS Synth Biol. 2014; 3:353. [PubMed: 24200191]
21. BSA has 59 lyssine residues of which 30–35 are on the surface or in pockets available for labeling, as noted in: Koniev O, Wagner A. Chem Soc Rev. 2015; 44:5495. [PubMed: 26000775] Hirayama K, Akashi S, Furuya M, Fukuhara KI. Biochem Biophys Res Commun. 1990; 173:639. [PubMed: 2260975] Wurm F, Steinbach T, Klok HA. Chem Commun. 2013; 49:7815.
22. Huang BX, Kim HY, Dass C. J Am Soc Mass Spectrom. 2004; 15:1237. [PubMed: 15276171]
23. Risk BA, Edwards NJ, Giddings MC. J Proteome Res. 2013; 12:4240. [PubMed: 23875887]
24. a) Na S, Bandeira N, Paek E. Mol Cell Proteomics. 2012; 11:M111.010199.b) Horlacher O, Lisacek F, Müller M. J Proteome Res. 2016; 15:721. [PubMed: 26653734]
25. Álvarez-Micó X, Rocha DD, Guimarães LA, Ambrose A, Chapman E, Costa-Lotufo LV, La Clair JJ, Fenical W. ChemBioChem. 2015; 16:2002. [PubMed: 26267855]

26. Mellert K, Lamla M, Scheffzek K, Wittig R, Kaufmann D. PLoS One. 2012; 7:e52473. [PubMed: 23285056]

27. a) Mai TD, Pereiro I, Hiraoui M, Viovy JL, Descroix S, Taverna M, Smadja C. Analyst. 2015; 140:5891. [PubMed: 26206107] b) Witte MD, Wu T, Guimaraes CP, Theile CS, Blom AE, Ingram JR, Li Z, Kundrat L, Goldberg SD, Ploegh HL. Nat Protoc. 2015; 10:508. [PubMed: 25719269]

28. Saito I, Shibata E, Huang J, Hisanaga N, Ono Y, Takeuchi Y. Br J Ind Med. 1991; 48:568–574. [PubMed: 1878315]

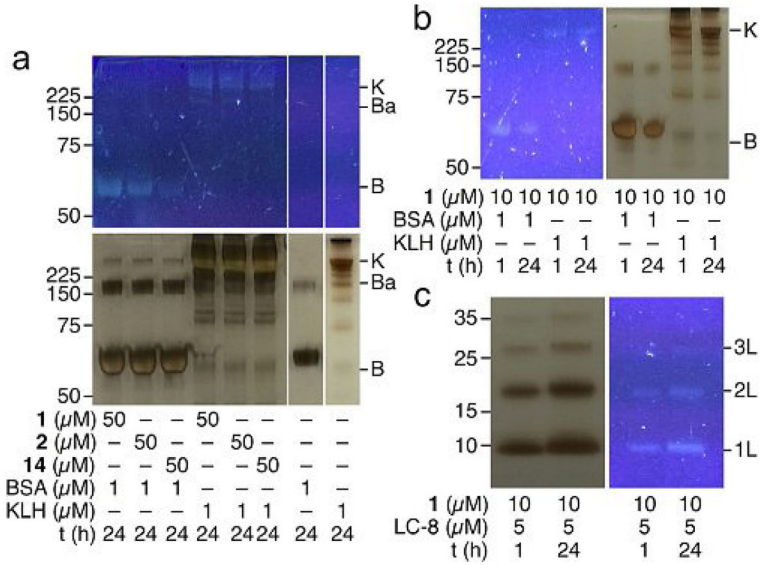


Figure 1.

The Paal–Knorr labeling of BSA, KLH, and LC-8. a) The labeling of BSA or KLH in PBS (pH 7.2) with diketone probes **1** and **2** as compared to monoketone control **14**. The gel was visualized on a UV light at 280 nm (top) and then silver stained (bottom). b) The limit of visual detection by SDS-PAGE was approximately 1 μg of protein bearing >1 label per protein.[17] C) The labeling of LC-8 with probe **1** at 1 h or 24 h. B, K, and L denote BSA, KLH, and LC-8, respectively. Concentrations are provided in micromolar. One microgram of protein was loaded per lane, with the exception of the control lanes (right two lanes in Figure 1 a where 0.5 μg was loaded). Aggregate bands were observed from BSA (Ba). KHL was obtained commercially, and the additional bands observed during silver staining are not atypical, as it is purified from natural sources.

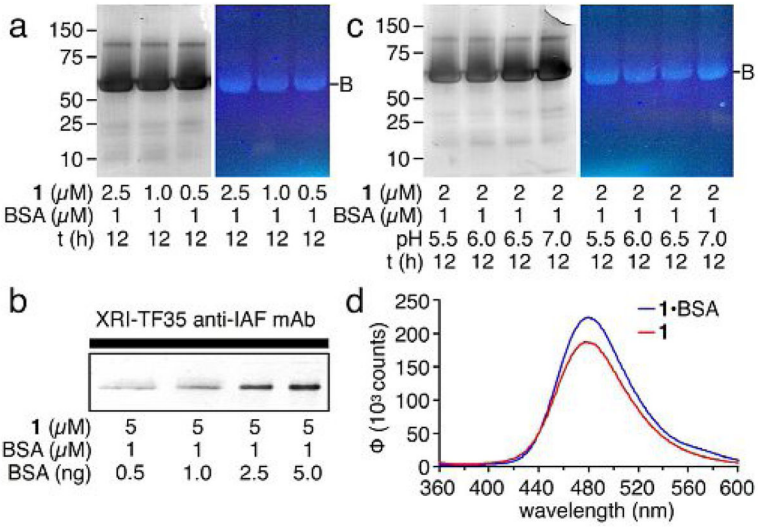


Figure 2.

Paal–Knorr labeling of BSA. a) Labeling of BSA can be detected down to 0.5 equiv. of probe **1** when scanned on a Typhoon laser scanner (Amersham). b) Western blot analyses can be used to further reduce the detection level of BSA labeled with **1**. c) Evaluation of pH effects on the labeling of BSA by probe **1**. Concentrations are provided in micromolar, and ≈ 1 μg of protein was loaded per lane. d) Fluorescence (*Φ*) spectra of probe **1** (10 μM) and BSA·**1** prepared by treatment of BSA (1 μM) with probe **1** (5 μM) for 12 h in PBS (pH 7.2). TLC and LC-MS analysis indicated that samples of BSA·**1** were free of unreacted probe **1**.

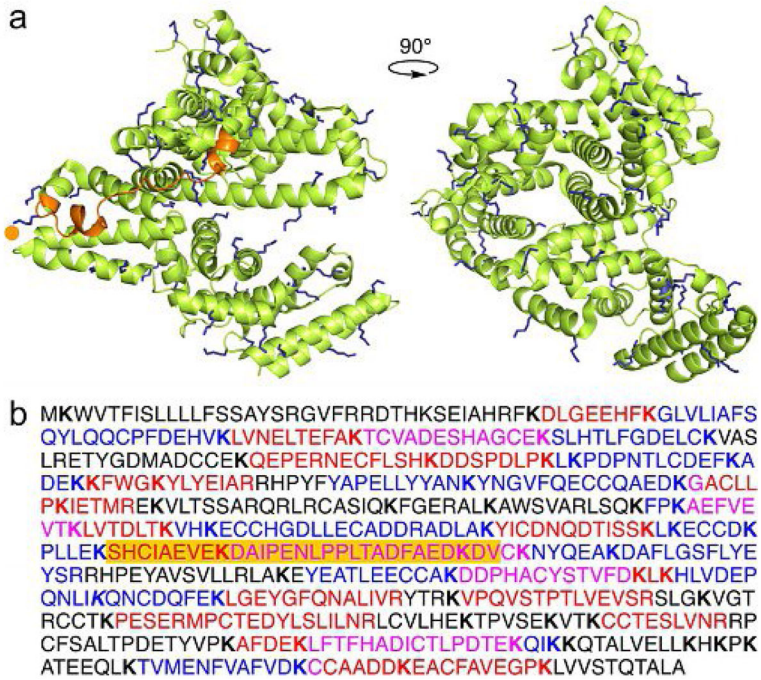


Figure 3.

Labeling of BSA with probe **1**. a) Each molecule of BSA has 59 lysine residues, 30–35 of which have primary amines that are capable of being conjugated.[21] b) Peptide coverage map for BSA labeled with detected peptides in red or blue and overlapping regions of these peptides in magenta. LC-MS/MS analysis confirmed the modification of Lys336 (orange dot) within an observed peptide (highlighted in orange). Observation of the peptide in (b) does not suggest that labeling occurred on a specific lysine residue, rather, we were able to detect labeling at this position.

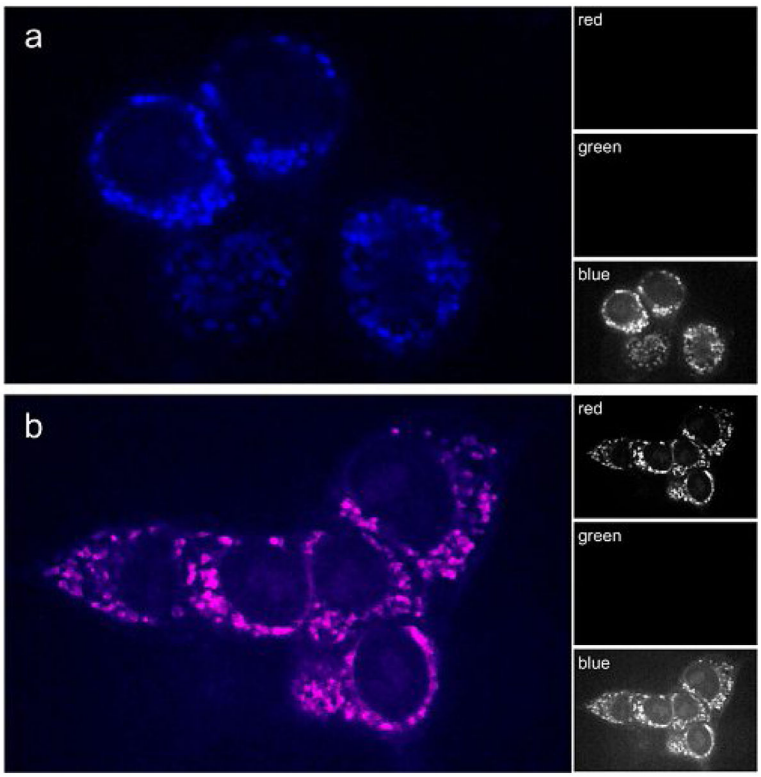
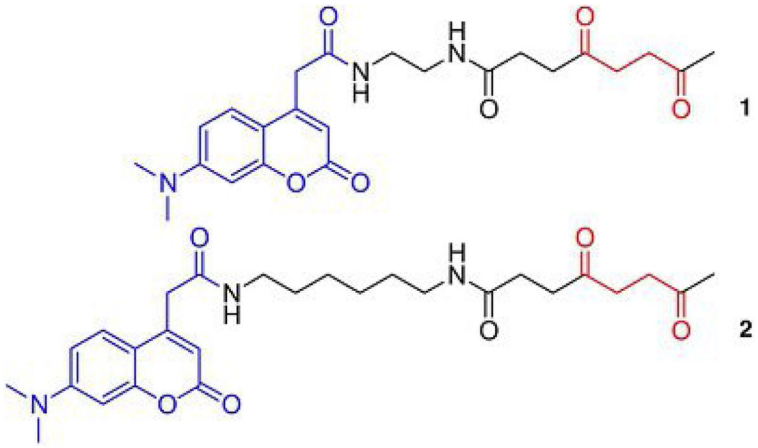


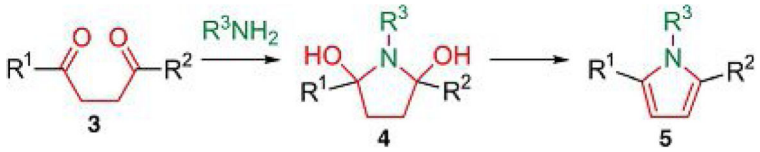
Figure 4.

Cell imaging of the uptake of **1**-labeled BSA. a) Image of HCT-116 cells stained with **1**-labeled BSA (blue). HCT-116 cells grown to 10^5 cells cm^{-2} were incubated for 3 h in media containing **1**-labeled BSA (25 μM). After this period, the cells were washed with media and imaged live. b) Image of HCT-116 cells in (a) after incubation with LysoTracker Red DND-99 (2.5 μM) for 15 min prior to imaging.[23] Images were collected with blue (405 nm laser with emission filtered at (447 ± 60) nm); green (543 nm laser with emission filtered at (593 ± 40) nm) and red (633 nm laser with emission filtered at (692 ± 40) nm) fluorescence. Overlay image is provided at left, and selected channels are shown at the right of each panel. We confirmed the presence of **1**-labeled BSA in the treated cells using western blot analyses, as shown in Figure S1.



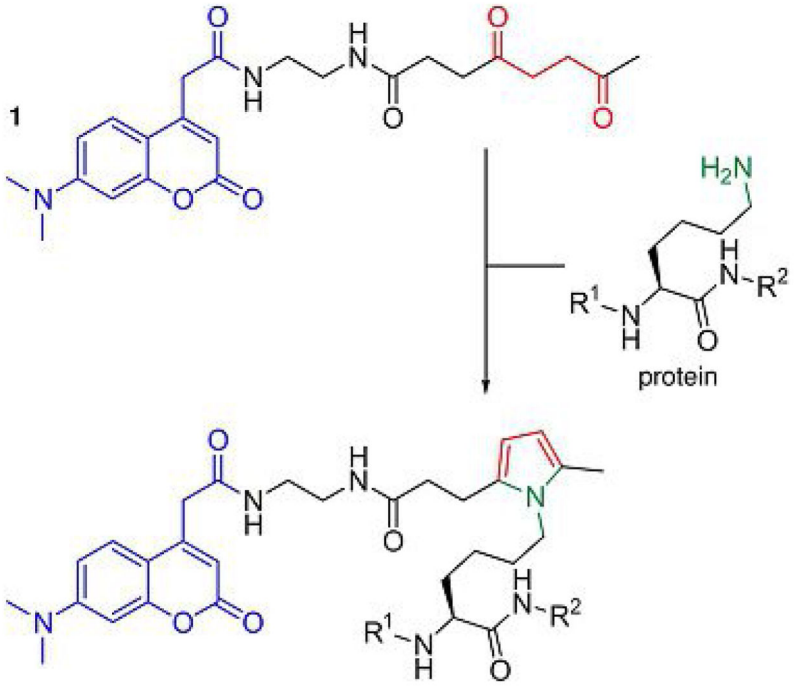
Scheme 1.

Structures of Paal–Knorr probes **1** and **2**. The 7-dimethylamino-4-coumarin or IAF tag and 1,4-diketone warhead are colored blue and red, respectively.



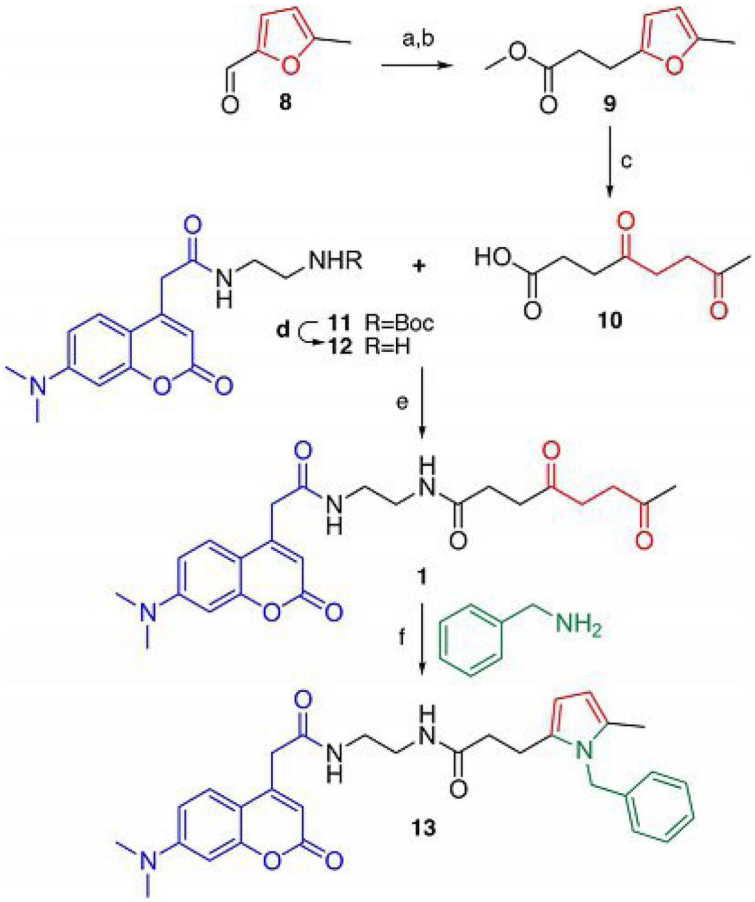
Scheme 2.

The Paal–Knorr reaction. Diketone **3** condenses with an amine to generate hemiaminal **4**, which undergoes a facile double dehydration to afford pyrrole **5**. The 1,4-diketone and amine moieties are colored red and green, respectively.



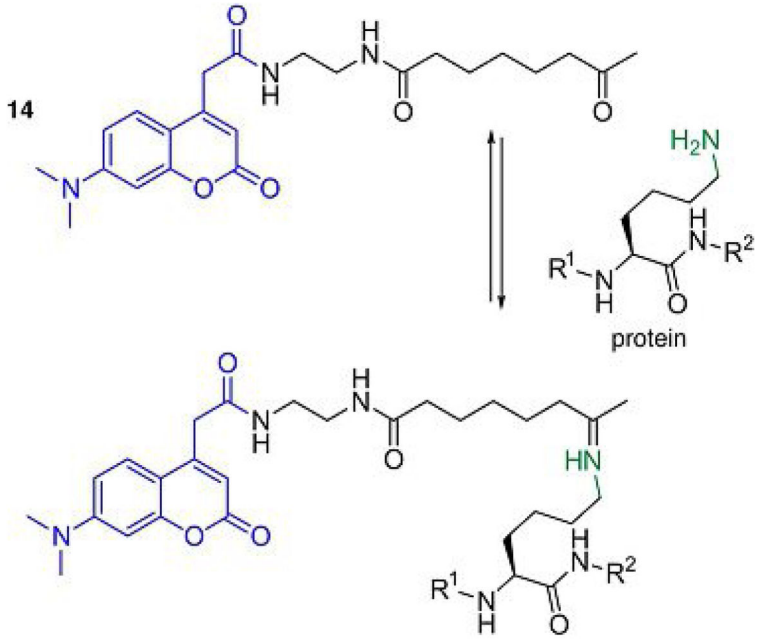
Scheme 3.

A schematic representation of the Paal–Knorr labeling of a protein with probe **1**. The IAF tag, 1,4-diketone, and amine are colored blue, red, and green, respectively.



Scheme 4.

Synthesis of Paal–Knorr probe **1**. a) $Ph_3P = CHCO_2Me$, THF, reflux, 20 h, 92 %; b) H_2, Pd/C, EtOH, RT, 24 h, 78 %; c) H_2SO_4 (trace), 50 % aq. HOAc, 110 °C, 15 h, 62 %; d) 0.5 MHCl, 1,4-dioxane, CH_2Cl_2, RT, 2 h, 98 %; e) EDAC, DMAP, DMF, CH_2Cl_2, 20 h, 86 %; f) AcOH, THF, RT, 5 h, 86 %. The IAF tag, 1,4-diketone, and amine are colored blue, red, and green, respectively.



Scheme 5.

A schematic representation of Schiff base formation with control **14**. The IAF tag and amine are colored blue and green, respectively.