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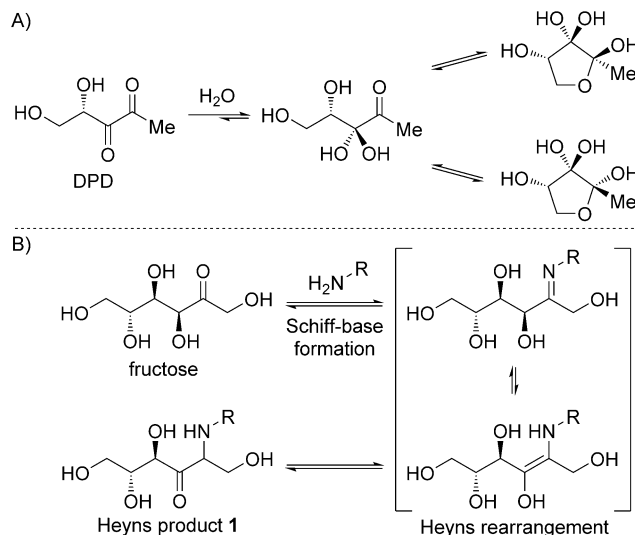
Glycation Reactivity of a Quorum-Sensing Signaling Molecule

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Abstract: Reported herein is that (4S)-4,5-dihydroxy-2,3-pentanedione (DPD) can undergo a previously undocumented non-enzymatic glycation reaction. Incubation of DPD with viral DNA or the antibiotic gramicidin S resulted in significant biochemical alterations. A protein-labeling method was consequently developed that facilitated the identification of unrecognized glycation targets of DPD in a prokaryotic system. These results open new avenues toward tracking and understanding the fate and function of the elusive quorum-sensing signaling molecule.

Over the past decade, our group and others have investigated the bacterial biochemical properties of (4S)-4,5-dihydroxy-2,3-pentanedione (DPD).^[1] Our interest in this molecule originally stemmed from its distinct densely oxygenated five-carbon-atom makeup, and reports touting its ability to coordinate gene expression in both gram-negative and gram-positive bacteria in a population-dependent manner, termed as autoinducer-2 quorum sensing (AI-2 QS). Moreover, we have shown that DPD can exist in a complex, multispecies equilibrium whereby its unique architecture allows it to undergo cyclization and ring-opening reactions in a similar fashion as do saccharides (Scheme 1 A).^[2]

Enormous resources have been invested in AI-2 QS trying to connect QS pathogenic events, however, these research efforts have only yielded sporadic reports of AI-2-QS-regulated behaviors.^[3] Fundamental to AI-2 QS on one hand is DPD's biosynthetic manufacturer present in over 70 species of bacteria, yet, a consistent link between the DPD signal, receptor, mechanism, and target output within this plethora of bacterial species has been lacking.^[4] Additional questions on DPD's role in cross-species signaling have also been dampened by the view that the luxS gene, which was thought to be dedicated to AI-2 production, also assumes an integral function in the activated methyl cycle.^[5] Taken together, while DPD has been portrayed as being central to



Scheme 1. A) DPD and its equilibrium species in aqueous solution. B) Fructose undergoing the Heyns rearrangement with lysine.

bacterial interspecies quorum sensing, its importance in bacterial pathophysiological control remains elusive. Herein, an additional piece to this molecule's yin and yang chemical biology is presented. We describe an intrinsic reactivity of DPD a reactivity that enables DNA- and protein-glycation, a previously undocumented biochemical role for this signaling molecule in the bacterial cell.

It has been established that the carbonyl moiety of a reducing sugar is capable of reacting non-enzymatically with the side chains of lysine/arginine residues embedded within a protein (Maillard reaction), and is initiated by the formation of a transient Schiff base. This Schiff base intermediate can then undergo an Amadori (in aldoses) or Heyns rearrangement (in ketoses; Scheme 1 B), which can ultimately lead to the formation of advanced glycation end products (AGEs),^[6] and subsequent alteration of the protein's normal function. As such, AGEs have been implicated in a number of pathologies associated with aging, diabetes, and arthritis.^[7] Research findings demonstrating that nucleotide bases could also participate in advanced glycosylation reactions have offered additional insights into the pathophysiology of AGE-related diseases. Prior studies have shown that DNA-modification by glucose 6-phosphate (G-6-P) leads to a significant decrease in transfection efficiency and an increase in plasmid mutations *in vivo*.^[8] Incorporation of the stable adduct *N*²-1-(carboxyethyl)-2'-deoxyguanosine (which can form on prolonged incubation of DNA with glucose or methylglyoxal) within template DNA blocked polymerase activity and

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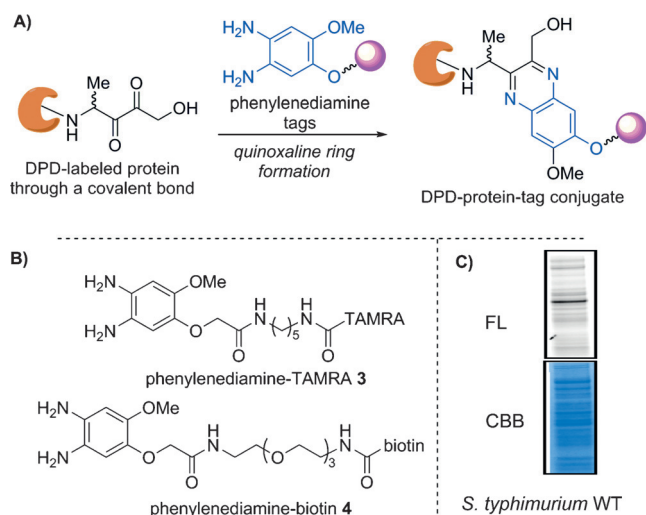


Figure 1. A) Labeled protein by quinoxaline ring formation. B) Phenylenediamine-TAMRA (3) and Phenylenediamine-biotin (4) tags. C) Labeling of proteome from wild-type *S. typhimurium*. FL = fluorescence.

tags for further studies: phenylenediamine-TAMRA (3) and phenylenediamine-biotin (4; Figures 1B; see Figure S6 in the Supporting Information). The advantage is that the probes could be trapped, as either a protein-DPD-TAMRA or protein-DPD-biotin conjugate, and could be analyzed by in-gel fluorescence or subjected to affinity purification, respectively, thus providing invaluable information regarding which proteins rapidly undergo DPD glycation.

Previously, glycation involving common saccharides was only considered to be relevant for eukaryotic organisms as it is chemically challenging (taking weeks to occur). However, Ivanov and co-workers provided evidence that AGE formation also occurs in prokaryotes despite its short life span.^[15] As a first demonstration of the utility of the probes to examine DPD-linked protein glycation, we set out to label the whole lysate derived from the wild-type *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain 14028, as DPD's role in the AI-2 QS of *S. typhimurium* has been well documented.^[1a,c,d] This strain was grown on a stationary phase ($OD_{600} = 3.6$) and the whole cell lysate was incubated with DPD (4 mM) at pH 7.0 at 25 °C for 1 hour. After protein precipitation, the protein pellets were re-solubilized and then incubated with phenylenediamine-TAMRA (3; 100 μ M) at 25 °C for 1 hour. As shown in Figure 1C, a comparison of the fluorescence image with the Coomassie brilliant blue (CBB) reveals that the proteins in this milieu are rapidly glycosylated. Importantly, when the lysate was incubated without DPD, no labeling was observed, thus indicating that the phenylenediamine tag is selective for DPD-glycated proteins (see Figure S7 in the Supporting Information).

Encouraged by these results, we sought to identify which proteins are glycosylated by DPD. As such, we used a combination strategy utilizing phenylenediamine-biotin (4) and mass spectrometry-based proteomic-profiling techniques.^[16] Here, the whole-cell lysate (*S. typhimurium* strain 14028) was used either with or without denaturing prior to DPD labeling

(denatured or native experiment, respectively). We anticipated that a comparison of these two samples would enable us to determine which proteins are modified by DPD, and thus possess the specific structural interactions necessary to form the Heyns-protein adduct. Labeled proteins were enriched using streptavidin beads, trypsinized, and analyzed by tandem ESI-MS/MS (see the Supporting Information for protocols). To exclude statistically irrelevant hits, positive hits were defined based on the criteria as follows: 1) a minimum of two unique peptides per protein in all three replicates, 2) p value < 0.05, and 3) greater than twofold more spectral intensity detected in the native sample than in the denatured sample.^[17]

In the native sample, 63 proteins met the criteria for positive hits (see Table S2 and Figure S8 in the Supporting Information). Interestingly, several are known to regulate AI-2 QS and the *lsr* repressor protein LsrR is one such example. According to previous biochemical studies^[1d,18] and crystallographic data of LsrR derived from *Escherichia coli*,^[19] it is the phosphorylated form of DPD (phospho-DPD) which binds to this protein, thus provoking destabilization and derepression of the *lsr* operon. However, it has been proposed that intact linear DPD can also bind to LsrR, thus resulting in altered biofilm architecture through a distinct signaling pathway in *E. coli*.^[20] In addition, it is noteworthy that the periplasmic protein LsrB, which recognizes and internalizes the AI-2 signal,^[1c] was not detected as a positive hit. However, it is the cyclic form of DPD that LsrB recognizes, and thus accounts for the lack of reactivity in this labeling.

Other positive hits in the above-mentioned proteome labeling included the phosphoenolpyruvate phosphotransferase PT1^[21] and phosphoenolpyruvate synthase regulatory protein PsrP. These comprise the phosphoenolpyruvate phosphotransferase system (PTS), which is required for initial internalization of AI-2 signals into the cytosol, followed by *lsr* operon activation.^[22] In addition, the NAD(P)H-dependent glycerol-3-phosphate dehydrogenase GpsA was detected. This protein reduces dihydroxyacetone phosphate, which inhibits the regulatory activity of LsrR.^[23]

To gain a greater appreciation of DPD-protein labeling efficiency, LsrR from *S. typhimurium* LT2 was cloned and overexpressed to afford a whole-cell lysate, and to this, BSA was added as a control protein, as its use as a glycation model is well documented.^[24] The obtained protein solution was labeled and visualized with DPD and 3 as described previously (Figures 2, see Figure S7 in the Supporting Information). As expected, labeling of LsrR was dependent on concentration, temperature, and incubation time (Figures 2A,B). Labeling efficiency was reduced when LsrR was denatured prior to incubation with DPD (Lanes 2 and 3 in Figure 2C). Quite remarkably, the unnatural (4*R*)-DPD (*ent*-DPD) exhibited significantly reduced labeling efficiency (Lanes 2 and 4 in Figure 2C). In contrast, labeling of BSA was marginal compared to that of LsrR, suggesting that the former does not structurally recognize DPD, and thus underlining the selectivity of our labeling probe. To rule out additional spurious artifacts, the specificity of the phenylenediamine probe was examined using fructose and glucose instead of DPD, and it did not give fluorescently labeled LsrR (see Figure S9 in the Supporting Information). Collectively,

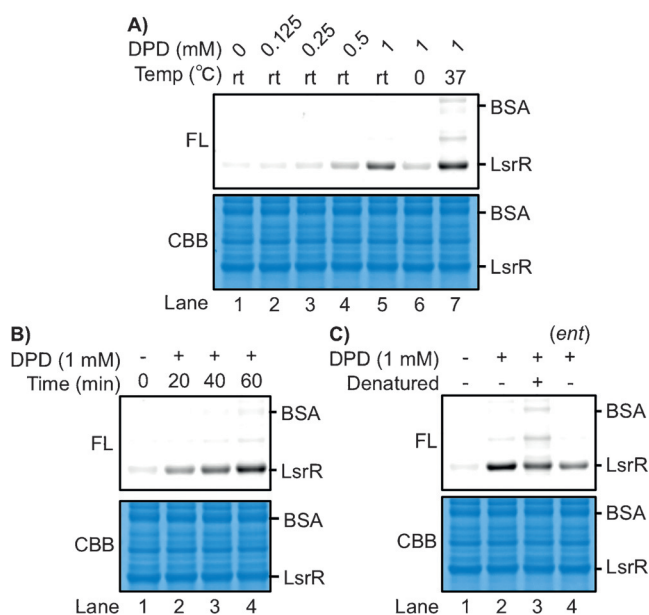


Figure 2. DPD labeling of LsrR under various conditions: A) concentration of DPD and incubation temperature. B) Incubation time. C) Denatured LsrR and *ent*-DPD.

our labeling method allows selective detection of proteins which could recognize and react with DPD. These results also illustrate that the protein must possess a unique tertiary structure microenvironment for Heyns-DPD protein marking to transpire. In particular, the observed stereoselectivity is in contrast with protein glycation by natural sugars, where reactivity is not dictated by stereochemistry.^[25]

The significance of these proteomics findings to bacterial physiology must be viewed with caution. In our labeling experiments, we used millimolar concentrations of DPD, concentrations which may raise questions for its physiological relevance. Quantitative detection of QS molecules in real time is a rather challenging task as they are produced at different concentrations throughout the bacterial life cycle. AI-2 has been reported to accumulate at a maximal intracellular level of 2 mM in *E. coli*.^[26] However, we speculate that it could exist at a higher concentration given the transient and, as our current data imply, highly reactive nature of DPD. Although our results offer the tantalizing possibility that DPD acts as a post-translational modifier for regulation of AI-2 QS, the fortuity that this biochemical processing is non-QS-2-related could not be discounted and remains to be demonstrated. What is clear is that DPD has the hallmarks of a glycosylating agent and we have disclosed evidence characterizing this previously unrecognized reactivity. Using ssDNA and gramicidin S as model systems, we have shown that DPD can react with both to produce conspicuous biochemical alterations. We subsequently developed a labeling method that enabled the explicit identification of proteins which can recognize and react with DPD, and as a first application, successfully labeled a prokaryotic proteome. A fascinating aspect is the rapid and stereospecific nature of DPD-induced glycation, requiring bacterial proteins to possess a requisite tertiary structure microenvironment for the DPD–Heyns

reaction to ensue. Future studies from our group will focus upon unraveling the chemical makeup of these Heyns products as well as the structural requirements and downstream effects of DPD glycation. More importantly, we are endeavoring to expand the utility of our labeling probes in eukaryotic systems, as this will provide further insights into the physiological implications of the quorum-sensing signaling molecule beyond quorum sensing.

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