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Structural Requirements of 2-Oxoglutaric Acid Analogues To Mimic Its Signaling Function

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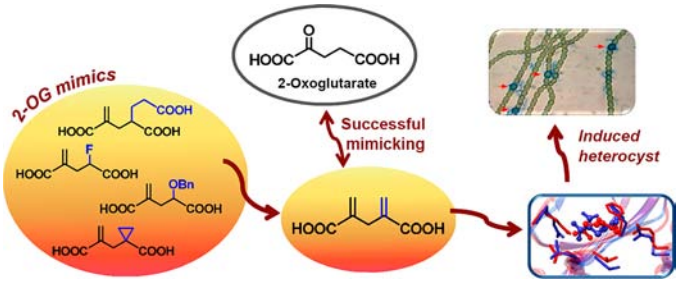
ABSTRACT



A 2-oxoglutaric acid (2-OG) probe bearing a methylene group introduced at the C4 position and a vinyl group to replace the carbonyl group at the C2 position elicited characteristic affinity for NtcA, the 2-OG receptor, while maintaining the signaling function of the parent natural metabolite 2-OG. This discovery opens new perspectives in the design, synthesis, and implementation of specific 2-OG analogues as molecular probes for investigating the complex 2-OG signaling pathways.

Derived from the Krebs cycle, the metabolite 2-oxoglutaric acid (2-OG, Scheme 1) is a strategically important precursor for biomolecular synthesis and cellular energy production. In addition to these traditional roles, it recently regained considerable attention as a key signaling molecule[1−4] in many other biological processes in various organisms.[5−9] Studies with cyanobacteria *Anabaena* sp. PCC7120 (hereafter referred to as *Anabaena*) consitute an exiquisite example: in *Anabaena*, the accumulation of 2-OG constitutes a nitrogen starvation signal which triggers a cascade of cellular responses ultimately leading to the formation of heterocysts.[3,10] Studying the signaling roles of 2-OG *in vivo* is a grand challenge since this compound is rapidly metabolized. We inventively used

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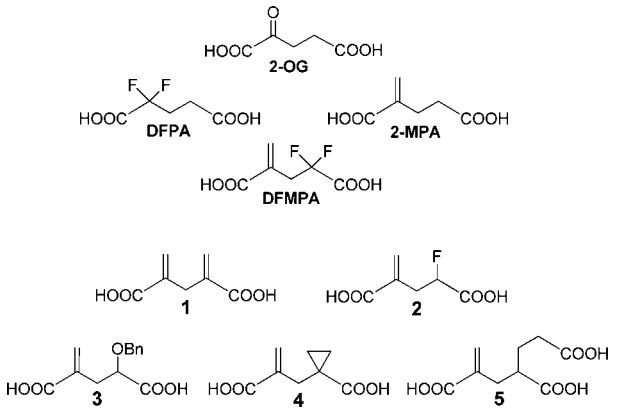
the fluorinated nonmetabolisable 2-OG analogue DFPA (Scheme 1) to mimic 2-OG, thus demonstrating for the first time the signaling role of 2-OG *in vivo* in *Anabaena*.[3] We then showed that another 2-OG analogue, featuring a planar vinyl group to mimic the carbonyl group in 2-OG (2-MPA, Scheme 1), was also able to play a similar role in *Anabaena*. These findings led to us conclude that it is the planar ketone form rather than the tetrahedral ketal form of 2-OG that is essential for the signaling activity of this molecule.[11] Recently, our group synthesized DFMPA (Scheme 1), a hybrid probe based on DFPA and 2-MPA carrying a vinyl and a *gem*-difluoromethylene group at the C2 and C4 positions, respectively. Surprisingly, DFMPA could also mimic the signaling function of 2-OG and successfully trigger the formation of heterocysts in cyanobacteria.[12] Although DFMPA shares with 2-OG some structural features such as two carboxylic acid terminals, it also presents essential chemical differences, particularly at the C4 position. The surprising yet interesting results obtained with DFMPA suggest that structural alterations at C4 in 2-OG analogues can be tolerated, leading to 2-OG mimics able to bind their receptor and, in so doing, exercise a regulatory function on the metabolic pathways in *Anabaena*.

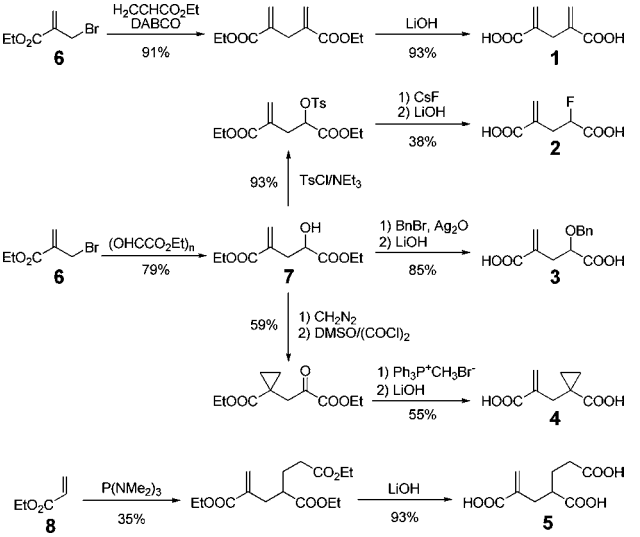
Probing the chemical modifications tolerated by 2-OG is interesting in studies on the biological role of 2-OG itself, and at the same time, such an investigation could also be of great help in clarifying the interaction of this metabolite with potential receptors, in providing useful information to design competent affinity probes for identifying new 2-OG receptors, and, ultimately, in dissecting the complex signaling pathways of 2-OG. We therefore further explored the structural requirement for 2-OG analogues to mimic the signaling role of 2-OG by specifically varying the substituents at its C4 position. Our previous work proved that replacing the carbonyl function at the C2 position of 2-OG with a vinyl group led to a metabolite derivative with unaltered signaling activity.[11] Therefore, we designed new compounds **1**–**5** in which the vinyl functionality at the C2 position was maintained while the position C4 was modified with a series of substituents illustrated in Scheme 1. The choice of chemical groups at C4 encompasses a diversity of chemical structures with varying nature, size, and shape which, in turn, allow us to explore the effects of structural variation and the requirement at the C4 position of mimicking the biological behavior of the original metabolite. The results stemming from this study will be instrumental in the design and synthesis of new 2-OG analogues. At the same time, and perhaps more importantly, we expect to use these analogues in the quest for unknown 2-OG receptors and in shedding light on the intriguing signaling pathways of 2-OG in cyanobacteria and other organisms.

Scheme 1. 2-OG and Its Structrural Analogues



The synthesis of **1**–**5** was successfully achieved according to the strategies presented in Scheme 2. Probe **1** was obtained using an established protocol described in literature,[13] whereas **2**–**4** were prepared starting from the precursor **7** which, in turn, was obtained by coupling **6** with poly(ethyl glyoxylate).[14] After tosylation of the hydroxyl group in **7** and subsequent nucleophilic substitution with CsF, the resulting product was hydrolyzed under alkali

Scheme 2. Synthesis of Probe **1**–**5**



conditions to afford **2**.[15] Probe **3** was obtained via direct benzylation of **7** followed by subsequent ester hydrolysis. To synthesize **4**, the cyclopropyl group was imported via carbene insertion onto the vinyl group in **7**; the hydroxyl group was then oxidized to a carbonyl group, which was further transformed into a vinyl group via Wittig reaction.

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(15) The poor solubility of CsF in organic solvent could be the main reason for the low yield of the nucleophilic substitution reaction.

Subsequent hydrolysis led to **4** in good yield. Finally, probe **5** was obtained by trimerization of **8** followed by simultaneous hydrolysis of all three ester groups into carboxylic acid functionalities.[16]

With probes **1**–**5** at hand, we next studied their ability to mimic 2-OG function in *Anabaena*, an excellent model to investigate the signaling role of 2-OG in nitrogen metabolism since it produces morphologically distinct heterocysts in response to combined-nitrogen depletion.[17] The so-formed heterocysts are able to fix N_2 from air, allowing *Anabaena* to survive even in the absence of combined nitrogen in the nutrition medium.[18] Importantly, since heterocysts differ morphologically from vegetative cells, they can be easily distinguished under a microscope. Furthermore, heterocyst differentiation can be repressed at early stages, when a combined nitrogen source such as ammonium or nitrate is supplied to the growth medium. Hence, we first examined the ability of **1**–**5** to induce heterocyst formation in the presence of high ammonium concentration (5 mM) which, as mentioned above, represents a strongly repressive condition to heterocyst formation. Note that, like the natural metabolite 2-OG, probes **1**–**5** are highly negatively charged; therefore, their passage across the cellular membrane should, at least in principle, be disfavored.[10] To address this issue, we constructed a recombinant strain of *Anabaena* expressing a heterologous 2-OG permease KgtP from *E. coli* (herein referred to as the KGTP strain) which can efficiently take up 2-OG and its analogues.[3,10–12,19] Notably, among all five probes, only **1** could trigger heterocyst formation (Figure 1) in a manner similar to that observed for DFPA, 2-MPA, and DFMPA.[3,11,12]

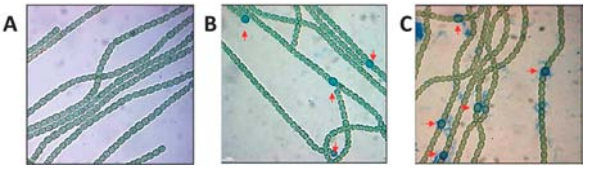

Figure 1. Induction of heterocyst differentiation under repressive conditions in the KGTP stain of *Anabaena* incubated (A) without any probe, and in the presence of (B) DFMPA and (C) **1**, respectively. Filaments were stained with alcian blue. Heterocysts are indicated by red arrows.

To understand the reason for the distinctive behavior of probe **1** in inducing heterocyst formation, we next examined the cellular uptake efficiency of all 5 probes. Uptake of **1**–**5** by the KGTP strain was monitored by inspecting the characteristic ^{1}H NMR signals associated with the vinyl groups around 5–6 ppm using ^{1}H High Resolution Magic Angle Spinning (HRMAS) NMR, an excellent nondestructive method for *in vivo* analysis of the metabolic profiles of whole cells.[20] Compared to DFMPA as the positive control, both **1** and **4** were efficiently taken up by the KGTP strain (Figure 2). Conversely, only a limited amount of **5** was internalized by cells, while no notable uptake was observed for either **2** or **3**. These findings allowed us to conclude that the poor/no cellular uptake of **2**, **3**, and **5** could be directly related to the failure of these compounds in inducing heterocyst formation.

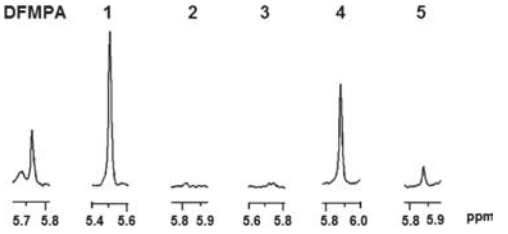

Figure 2. ^{1}H HRMAS NMR study on the cellular uptake of **1**–**5** in KGTP strain.

To unravel the molecular basis behind the singular behavior of **1** with respect to **4** in mimicking the signaling role of 2-OG and inducing heterocyst differentiation in *Anabaena*, we carried out an *in silico* study (see SI for details) on the binding of **1** and **4** to NtcA, a 2-OG receptor able to regulate nitrogen metabolism and responsible for heterocyst formation in cyanobacteria.[3,11,12,21] The key elements for a favorable binding of 2-OG toward NtcA involve a network of four hydrogen bonds between 2-OG and Phe75, Gly76, Val77, Leu78 of NtcA, respectively, together with the two carboxylic groups of 2-OG interacting with Arg88 and Arg129 in NtcA through permanent salt bridges (Figure S1). Interestingly, our results revealed that **1** adopts a binding pose within the NtcA binding pocket very similar to that of the natural ligand 2-OG (Figure 3A and C): indeed, three out of four hydrogen bonds are preserved and so are the two salt bridges involving the residues Arg88 and Arg129 although the corresponding H-bond interaction with the NH group of the peptide bond between Phe75 and Gly76 is no longer feasible due to the replacement of the carbonyl group of 2-OG by a vinyl moiety in **1**. These stabilizing interactions result in an elevated affinity of **1** toward NtcA (ΔG_{bind} = -14.0 ± 0.8 kJ/mol), similar to that of 2-OG (ΔG_{bind} = -15.5 ± 0.9 kJ/mol) (Table S1). The situation is quite different for compound **4** (Figure 3B and D): the two salt bridges between the two carboxylic groups of **4** and the guanidinium groups of Arg88 and Arg129 in NtcA are the only stabilizing interactions. Also, the presence of the cyclopropyl ring in **4** is found to induce an overall,

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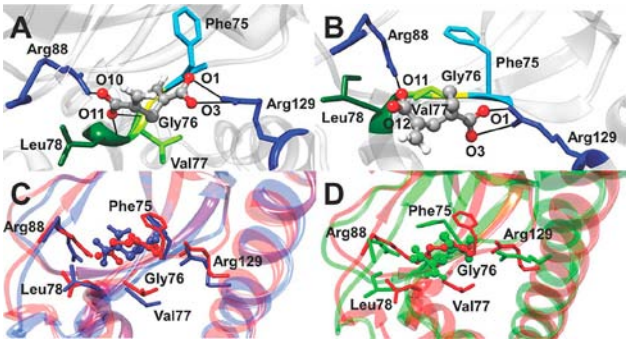

Figure 3. Equilibrated MD snapshot of **1** and **4** in complex with NtcA. Zoomed view of **1** (A) and **4** (B) within the NtcA binding pocket. Compounds **1** and **4** are shown as atom-colored balls-and-sticks (O, red; C, gray). The H-atoms of the methylene group(s) are highlighted in white. Black lines denote hydrogen bond and salt bridge interactions. NtcA residues directly involved in binding **1** and **4** are shown as labeled, colored sticks: Phe75 (light blue), Gly76 (yellow), Val77 (green), Leu78 (dark green), Arg88 and Arg129 (both in blue). (C) Superposition of NtcA/2-OG (red) and NtcA/**1** (blue) binding modes. (D) Superposition of NtcA/2-OG (red) and NtcA/**4** (green) binding modes. In all panels, water molecules, ions, and counterions are omitted for clarity.

considerable distortion of the whole binding site; particularly, the side chain of Phe75 is drastically rotated while Val77, Leu78, and Arg129 side chains all undergo a significant translational move. All these conformational readjustments result in a wider protein binding pocket and, ultimately, in less effective ligand/protein stabilizing interactions ($\Delta G_{bind} = -8.9 \pm 1.0$ kJ/mol). Thus, according to the computational results, even if compound **4** is able to cross the cellular membrane, its low affinity for the NtcA receptor reflects negligible biological activity.

To support the above *in silico* results, we further assessed **1** for its ability to promote DNA binding of NtcA. NtcA is the 2-OG receptor, which is also a transcription factor regulating nitrogen metabolism and initiating heterocyst formation in cyanobacteria. 2-OG enhances the binding affinity of NtcA toward its DNA promoter, an event which can be easily revealed by performing DNA mobility shift assays. Similarly to 2-OG and DFMPA, we observed that **1** could effectively promote DNA/NtcA binding (Figure 4). In contrast, **4** could not stably elicit DNA/NtcA binding (Figure S2). Taken together, our results provide a reasonable molecular rationale for the finding that only **1** among all the 2-OG analogues **1**–**5** designed and synthesized in this work can act as a regulator of NtcA and effectively mimic the signaling role of 2-OG.

In summary, we designed and synthesized novel 2-OG probes **1**–**5** bearing different moieties at the C4 position while retaining the vinyl group at the C2 position with the aim of understanding the structural requirements of 2-OG analogues at the C4 position in mimicking the signaling

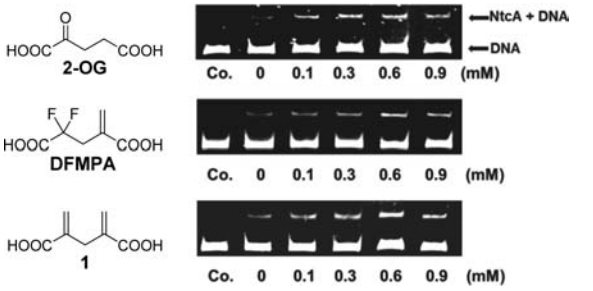

Figure 4. DNA biding affinity of NtcA at various concentrations of 2-OG, DFMPA, and probe **1**. Co: DNA fragment without NtcA.

role of 2-OG in cells. Our results revealed that only compound **1** is endowed with cell internalization capacity, NtcA target protein affinity, and heterocyst formation activity similar to the natural Krebs cycle metabolite 2-OG. Molecular modeling results demonstrated that **1** and 2-OG share similar binding modes within the NtcA binding pocket; thus, **1** is able to exert the same signaling function as 2-OG for heterocyst formation in cyanobacteria *Anabaena*. Collectively, our results allow us to conclude that the C4 position of 2-OG is of great importance and that either the vinyl or the *gem*-difluoromethylene group can be introduced at this position in order to retain the signaling function of 2-OG. These findings represent a further contribution toward a better understanding of the nature and mechanisms of 2-OG binding to the NctA receptor and will assist us in the design of new 2-OG probes. We also encourage and foresee the use of such analogues in the identification of new 2-OG receptors, with the ultimate, ambitious task of expanding our knowledge of the various signaling pathways of 2-OG such as the balance of carbon to nitrogen in available nutrients,[5,6] the role of 2-OG-dependent oxygenases in epigenetic regulation,[8] and the altered metabolism of 2-OG to generate the "oncometabolite" 2-hydroxyglutarate, the reduced form of 2-OG, in various cancers.[9] Currently, we are actively pursuing research in these directions.

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Supporting Information Available. Chemical synthesis, analytical data, NMR spectra, biological study, computer modeling as well as Figures S1 and S2, Scheme S1, Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.