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2-Difluoromethylene-4-methylenepentanoic Acid, A Paradoxical Probe Able To Mimic the Signaling Role of 2-Oxoglutaric Acid in Cyanobacteria

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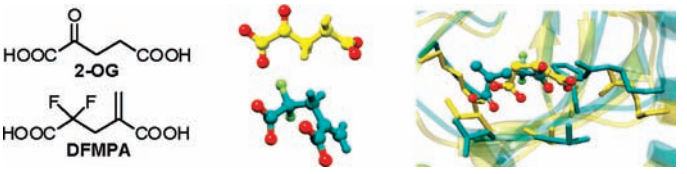
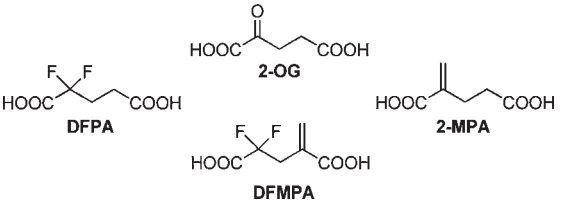
ABSTRACT



2-Difluoromethylene-4-methylenepentanoic acid (DFMPA), a seemingly deviated analog of 2-oxoglutaric acid (2-OG), could surprisingly mimic its signaling function in cyanobacteria. Computer modeling revealed the favorable binding of DFMPA toward the 2-OG receptor, NtcA, via mutual conformational changes, suggesting that structural alteration of 2-OG is tolerated for it to exercise its signaling role. This extremely useful finding could be exploited for the design of affinity probes with which to study new 2-OG receptors in related signaling pathways.

2-Oxoglutaric acid (2-OG, Scheme 1), a metabolite of the highly conserved Krebs cycle, plays important roles not only in metabolism, but also in the signaling of various organisms from bacteria[1] to plants[2] and animals.[3] 2-OG forms a carbon skeleton for the assimilation of ammonia and serves as a lever managing the balance between nitrogen and carbon metabolism.[4] Using a biological model of cyanobacteria *Anabaena* PCC 7120 (hereafter referred to as *Anabaena*) and a chemical probe of 2-OG, DFPA (difluoromethylene pentandioc acid, in Scheme 1), we revealed the role of 2-OG as a nitrogen starvation signal *in vivo*.[5] With a difluoromethylene group replacing the carbonyl moiety, DFPA resembles 2-OG in structure and is stable to metabolization *in vivo*. Most importantly,

Scheme 1. 2-OG and Its Analogs DFPA, 2-MPA, and DFMPA (a hybrid of DFPA and 2-MPA)



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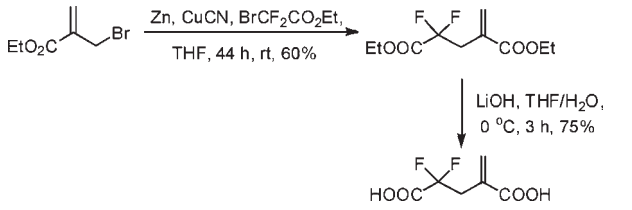
DFPA can mimic 2-OG, triggering the formation of nitrogen-fixing heterocysts and inducing a series of cellular responses by readjusting metabolism in *Anabaena*.[5] Using another nonmetabolizable probe, 2-MPA (2-methylenepentanoic acid, Scheme 1), which employs a vinyl group to mimic the ketone carbonyl function in 2-OG, we were able to confirm that it is the ketone form and not the ketal form of 2-OG that plays a signaling role during nitrogen starvation in *Anabaena*.[6] Subsequent structure/activity relationship analysis with different analogs bearing various functional groups to mimic the carbonyl group in 2-OG underlined the importance of this group in its signaling function.[6] Only closely resembling structural motifs such as difluoromethylene and vinyl moieties could replace the carbonyl group to ensure the signaling function of 2-OG in *Anabaena*, with DFPA and 2-MPA being the best examples.[6]

Spurred on by curiosity, we then developed a hybrid probe based on DFPA and 2-MPA, namely, DFMPA (2-difluoromethylene-4-methylenepentanoic acid, Scheme 1) for the structure/activity relationship study. DFMPA contains both difluoromethylene and vinyl groups at the C2 and C4 positions, respectively. The dual presence of these two structural motifs would appear to considerably change the structure of DFMPA compared to 2-OG. Consequently, it was expected that DFMPA would neither resemble 2-OG nor mimic its signaling role. However, surprisingly DFMPA could mimic the signaling function of 2-OG to induce heterocysts in *Anabaena*, in a way similar to both DFPA and 2-MPA, thus suggesting that structural alteration may be tolerated for 2-OG to exercise its signaling role. In order to understand this, computer modeling was carried out to investigate the interaction between DFMPA and NtcA, the 2-OG receptor which senses the nitrogen starvation status via changes in the level of 2-OG in *Anabaena*.[5−8] Interestingly, DFMPA was favorably accommodated in the binding site of NtcA via mutual conformational adaptation, further confirming the ability of DMFPA to mimc 2-OG and execute its signaling role. Here, we present our data supporting DFMPA's ability to mimic 2-OG signaling in *Anabaena* and provide our rationale for the structural alteration on the basis of computer modeling.

Synthesis of DFMPA was achieved by coupling ethyl 2-(bromomethyl)acrylate with ethyl bromodifluoroacetate in the presence of activated zinc powder and CuCN,[9] followed subsequently by alkali hydrolysis (Scheme 2).

The crystal structure of DFMPA shows a bent backbone conformation, which differs from the extended structures adopted by 2-OG, DFPA, and 2-MPA (Figure S1, Supporting Information). This structural deviation can be easily understood when considering the chemical

Scheme 2. Synthesis of DFMPA



composition of DFMPA. However, due to crystal packing, the crystal structures in the solid state may differ from the corresponding structures in solution.

We next studied the ability of DFMPA to mimic the 2-OG signaling function in *Anabaena*. This cyanobacterium is 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 deprivation.[10] These heterocysts are able to fix nitrogen from air and hence allow *Anabaena* to survive even under nitrogen depletion.[11] Importantly, since heterocysts differ morphologically from vegetative cells, they can be easily observed with light microscopy. Furthermore, heterocyst differentiation can be repressed when a combined nitrogen source such as ammonium or nitrate is present in the growth medium.

However, DFMPA cannot be taken up efficiently by *Anabaena* since it is negatively charged at physiological conditions.[12] We therefore used a recombinant strain of *Anabaena* expressing a heterologous 2-OG permease KgtP from *E. coli* (referred as KGTP) and which can efficiently take up 2-OG and its analogs.[5,6,12,13] The uptake of DFMPA in KGTP was studied using High Resolution Magic Angle Spinning ^{19}F and ^{1}H NMR (HRMAS NMR), an excellent nondestructive method for the *in vivo* analysis of the metabolic profiles of whole cells/tissues.[14] Surprisingly, DFMPA could be taken up by the KGTP strain in a similar way to DFPA and 2-MPA, as demonstrated by the clear signals observed for *gem*-difluoromethylene at −105 ppm in ^{19}F NMR (Figure 1A) and the corresponding vinyl group at 5.85 ppm in ^{1}H NMR respectively (Figure 1B), whereas, in the control experiments, no such NMR signals were produced by the KGTP strain or the wild type strain in the absence of DFMPA (data not shown). The effective uptake of DFMPA by the KGTP strain suggests its recognition by the 2-OG permease and consequential transporting across the cell membrane, thus implying that its resemblance with 2-OG may fool the 2-OG permease. Even more surprisingly was

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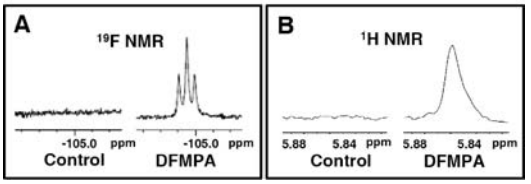

Figure 1. HRMAS NMR study on the uptake of DFMPA in the KGTP strain. (A) ^{19}F and (B) ^{1}H NMR spectra recorded in the absence (control) and presence of DFMPA.

the fact that DFMPA could also induce heterocyst formation (Figure 2) in the presence of NH_4^+, in a manner similar to DFPA and 2-MPA.[5,6] Collectively, these results suggest that DFMPA might mimic the signaling role of 2-OG in *Anabaena*.

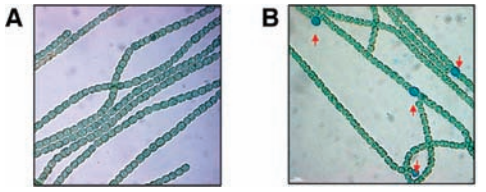

Figure 2. Induction of heterocyst differentiation under repressive conditions in the KGTP strain incubated in the absence (A) and presence (B) of DFMPA. Filaments were incubated with alcian blue, which specifically stains heterocyst envelope oly-saccharides (heterocysts are indicated by arrows).

To further confirm that DFMPA mimics the signaling role of 2-OG in *Anabaena*, we studied the effects of DFMPA on the DNA binding activity of the 2-OG receptor, NtcA,[5−7] a transcription factor which regulates nitrogen metabolism in cyanobacteria. NtcA is also essential for the initiation of heterocyst differentiation,[8] and 2-OG enhances the DNA binding affinity of NtcA (Figure 3A).[15] Similar to 2-OG, DFMPA promoted the DNA binding of NtcA with increasing concentrations (Figure 3B) thus implying its ability to mimic the signaling role of 2-OG via its binding to NtcA in *Anabaena*. Furthermore, no heterocyst differentiation was observed when DFMPA was incubated in the presence of ammonium with the -*ntcA* KGTP strain, a mutant in which no NtcA is expressed[15] (Figure S2, Supporting Information). Altogether, these data demonstrate that the heterocyst formation triggered by DFMPA in *Anabaena* requires the 2-OG receptor NtcA, in a way similar to 2-OG.

In light of these results, we wanted to know why DFMPA could maintain the signaling role of 2-OG despite its deviated structure and how DFMPA could bind toward the 2-OG receptor NtcA compared with 2-OG. It is important for us to understand the tolerance of structural alteration in 2-OG relating to its signaling role as it could offer us the opportunity to introduce functional groups in the design of affinity probes of 2-OG, which are essential in the identification of other new receptors with a view to constructing the signaling pathways of 2-OG. We therefore performed computer modeling to obtain detailed information on the putative binding modes of DFMPA to NtcA, with the aim of understanding the structural basis of the observed biological results.

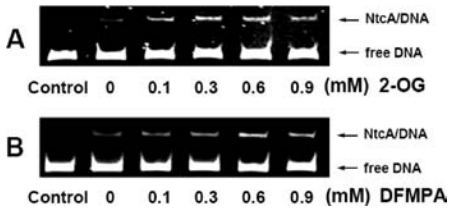

Figure 3. Effects of 2-OG (A) and its analog DFMPA (B) on the DNA binding activity of NtcA. Control: DNA fragment without NtcA.

The modeling procedure, including model building, protein docking, and free energy calculations, is based on the docking/Molecular Mechanics (MM)/Poisson−Boltzmann Surface Area (PBSA) approach[16] and is described in detail in the Supporting Information. Briefly, the procedure was developed and validated by comparing blank tests with the available X-ray structures of the 2-OG/NtcA and DFPA/NtcA complexes[7] (Figure S3, Supporting Information) and then applying these to DFMPA and 2-MPA, for which the crystal structures of their NtcA complexes are not available. Table 1 displays the estimated binding free energy (ΔG_{bind}) of DFMPA to the NtcA binding pocket, compared to the corresponding values for 2-OG and its analogs DFPA and 2-MPA. From this table we can see that not only is the applied computational strategy able to yield fairly accurate absolute free energies of the binding, as expected, but also, and perhaps more importantly, it can reproduce the trend exhibited by the corresponding biological activity determined for these compounds.

Table 1. Enthalpy (ΔH), Entropy ($-T\Delta S$), and Total Binding Free Energy (ΔG_{bind}) for 2-OG, DFPA, 2-MPA, and DFMPA in Complex with NtcA[a]

	2-OG	DFPA	2-MPA	DFMPA
ΔH	-50.7 ± 0.4	-49.6 ± 0.5	-48.3 ± 0.5	-46.9 ± 0.4
$-T\Delta S$	35.2 ± 0.8	36.0 ± 0.9	35.0 ± 0.8	36.5 ± 0.7
ΔG_{bind}	**-15.5 ± 0.9**	**-13.4 ± 1.0**	**-13.4 ± 1.0**	**-11.4 ± 0.8**

[a] All values are in kJ/mol. Errors are given as standard errors of the mean.

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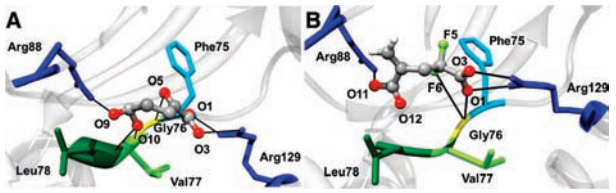

Figure 4. Binding of 2-OG (A) and DFMPA (B) in the binding pocket of NtcA. H-bonds and salt bridges are highlighted by black lines. The compounds are shown in atom-colored ball-and-sticks (C: gray; O: red; F: green). The two H atoms on the vinyl group are shown as white balls for visualization purposes. The protein residues directly involved in these interactions are as follows: Arg88 and Arg129 (blue), Phe75 (light blue), Gly76 (yellow), Val77 (light green), and Leu78 (dark green). All hydrogen atoms, water molecules, and counterions have been omitted for clarity.

Figure 4 shows two equilibrated molecular dynamics structures for 2-OG and DFMPA in complex with NctA. As we can see, the difluoromethylene group in DFMPA is located at the space occupied by the carbonyl group of 2-OG, whereas the vinyl group in DFMPA is comfortably accommodated within the NtcA binding pocket. A close analysis reveals that, for 2-OG (Figure 4A), the major contribution to NtcA binding stems from two salt bridges involving the side chains of Arg88 and Arg129 and a network of H-bonds among the main chains of residues Gly76, Val77, and Leu78. This efficient intermolecular interaction scheme is reflected in the affinity of 2-OG toward NtcA, as testified by the favorable value of the free energy of binding $\Delta G_{bind} = -15.5$ kj/mol, estimated using the MM/PBSA approach. In the case of DFMPA, the two salt bridge interactions with Arg129 and Arg88, crucial for the binding of 2-OG analogs to NtcA, have been maintained (Figure 4B), whereas the MD simulation gives no evidence for the interaction of DFMPA with Val77 or Leu78. Two stable H-bonds are however still detected between DFMPA and Gly76. Thus, the binding mode of DFMPA within the binding pocket of NtcA is quite similar to that adopted by 2-OG, although the number and strength of the intermolecular interactions between DFMPA and its target protein NtcA are slightly less, in agreement with the corresponding calculated free energy of binding (Table 1). These molecular modeling studies, coupled with the calculated affinity of 2-OG analogs toward NtcA, unequivocally support the apparently surprising experimental finding that DFMPA can mimic 2-OG via binding to the 2-OG receptor, NtcA, and thus exercise its signaling role in *Anabaena*.

It is important to mention that both DFMPA and the binding site of NtcA underwent conformational changes for mutually adapted and thus favorable interactions. DFMPA changed its conformation when complexed with NtcA (Figure S4D), similar to that observed for 2-OG on binding to NtcA[7] (Figure S4A). Considerable conformational changes were also observed for the NtcA binding site (Figures 4): Phe75 displayed a notable rotation of its phenyl ring, Arg129 underwent a significant translational move, and the main chain of Leu78, Val77, and Gly76 showed slight rearrangement. All these conformational changes allowed the favorable and comfortable binding of DFMPA in the NtcA binding pocket. Mutual adaptation or induced fit[17] is a common phenomenon observed in such as protein/DNA, protein/RNA, protein/protein, and protein/ligand complexes during their biological functions. Here, we have demonstrated that DFMPA also employs such a mechanism allowing its interaction with NtcA and assuring the signaling role of 2-OG. This finding is important as it implies that structural alteration at the C4 position can be tolerated in 2-OG for its signaling function via interaction with NtcA.

In summary, we have developed the hybrid probe DFMPA based on our previously developed 2-OG analogs, DFPA and 2-MPA. Our experimental results coupled with computer modeling have demonstrated that although DFMPA harbors both difluoromethylene and vinyl groups at the C2 and C4 positions simultaneously and has a seemingly important structural deviation compared to 2-OG, the signaling function of 2-OG is maintained, inducing heterocysts in *Anabaena* via the interaction with the 2-OG receptor, NtcA. Structural deviation of 2-OG is thus tolerated with respect to its signaling function. How strict alteration at C4 is allowed for mimicing the signaling role of 2-OG requires further investigation, for which we are actively working in this direction. The finding disclosed here that the tolerance of structural modification at the C4 position in 2-OG is extremely important and may offer us the opportunity to introduce functional groups in 2-OG with a view to designing affinity probes of 2-OG. They are essential means to identifying and studying other 2-OG receptors with the aim of constructing the signaling pathways of 2-OG and increasing our understanding of the signaling role of 2-OG.

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Supporting Information Available. Synthesis, NMR analysis, biological study, and computer modeling as well as Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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