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Surface-directed Selection of Dynamic Constitutional Frameworks as Optimized Microenvironment for Controlled Enzyme Activation

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ABSTRACT: Dynamic Constitutional Frameworks DCFs composed of cross-linked networks of imine-exchanging components have been generated and applied to the establishment of optimal microenvironments for carbonic anhydrase (CA) in aqueous solution. In response to the dynamic recomposition process, the enzyme showed distinct differential preferences for interchanging and incorporation of the amine functionalities, which were furthermore in good correlation with their inverse activation effects of CA. The results demonstrated surface-directed selection of the enzyme environment, where amines with lower enzyme-directed incorporation ratio possess higher activation effects, leading to a new strategy of self-optimization of the enzyme microenvironment for better catalytic performances.

Constitutional dynamic chemistry (CDC) is a powerful tool for component screening in complex systems and networks.¹ The systems are constructed using supramolecular interactions and reversible covalent bonds, generating dynamically interconverting architectures with adaptive behavior.²⁻³ In the presence of external stimuli, such as target biomolecules, optimal constituents can be selected and amplified, leading to various applications in organic synthesis,⁴⁻⁵ catalyst screening,⁶⁻⁷ drug discovery,⁸⁻⁹ materials design,¹⁰⁻¹¹ etc.

Enzymes constitute one of the most frequently used stimuli and have been demonstrated to play important roles in selection processes of constitutional dynamic systems.¹²⁻¹³ For example, lipases have been extensively employed as catalysts to perform secondary transformations, irreversibly converting optimal dynamic covalent substrates to the corresponding non-exchanging products, resulting in dynamic covalent resolution with good yields and high chemo-, regio- and enantio-selectivities.¹⁴⁻¹⁶ However, in most of these cases, the selection effect occurs in response to binding and processing inside the enzyme active sites, whereas selection based on weak interactions at the enzyme surfaces has not been widely explored.

The microenvironment surrounding the enzyme is known to be critical to the enzyme activity and selectivity.¹⁷ In our previous studies, dynamic constitutional frameworks-DCFs with multiple amide groups have been demonstrated to interact with enzymes, such as carbonic anhydrase (CA, EC 4.2.1.1) and lipases (EC 3.1.1.3), through H-bonding.¹⁸⁻²⁰ By forming protecting layers of DCFs around the target proteins, both the structural stability and activity of the enzymes could be clearly improved. However, the identification of the *fittest DCF environment* that may trigger the best catalytic performance for a specific enzyme remains an important challenge. One-by-one tests are not only time-consuming but also limited in detection accuracy and structural variety when compared to the *in situ* screening from the complex biocatalytic environment. This has been addressed in the present study, where we present a new concept of systemic, target-mediated self-optimization. Using a model system, the enzyme-

driven selection of an optimal environment in a constitutional dynamic screening process of reversible imine structures was achieved. Carbonic anhydrase could thus act as its own microenvironmental optimizer, in consequence to surface interactions between the enzyme and the dynamically formed DCFs (**Figure 1**).

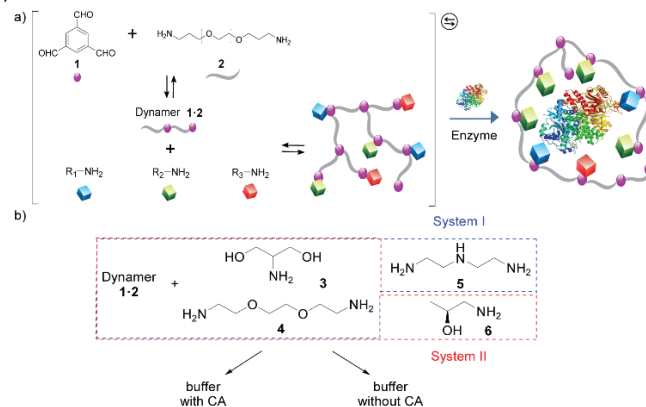


Figure 1. a) General concept of enzyme-driven microenvironment optimization of dynamic constitutional framework imine system; b) construction of dynamic systems.

In order to build the dynamic constitutional framework systems, an imine dynamer (**1-2**) was first synthesized from two components: 1,3,5-benzenetricarboxaldehyde (**1**) and bis(3-aminopropyl)-terminated poly(ethylene glycol) (**2**, $M_n \sim 1500 \text{ g}\cdot\text{mol}^{-1}$), in a molar ratio of 1:1 (**Figure 1a**). Trialdehyde **1** served here as the core structure linking the dynamic framework connectors, while leaving space for additional imine bonds between free aldehyde groups and additional amines. Connector **2** was chosen for its good water solubility, and for providing a flexible backbone preventing the imine functionalities from undergoing hydrolysis.¹⁸

The resulting dyanmer **1-2** was subsequently subjected to further functionalization with additional amines **3-6** (Figure 1b).

When it comes to small molecule-CA interactions, both enzyme inhibition and activation can be considered. However, the inhibitors usually act in a confined space within the enzyme active site,²¹⁻²³ while the activators operate at the entrance of the active site to facilitate the proton relay.²⁴⁻²⁵ In the current study, the large polymeric structures cannot easily enter the active sites as inhibitors. In addition, the dynamic systemic approach to identify optimized enzyme microenvironments can lead to higher catalytic performances of CA. Thus, different amines were chosen for the screening system as potential CA activators, and the corresponding DCFs exhibited CA activation effects.

Initially, three amines, including 2-amino-1,3-propanediol (**3**), 1,2-bis(2-aminoethoxy)ethane (**4**) and diethylenetriamine (**5**) were added together with dyanmer **1-2** at a concentration of 10 mM in D₃PO₄-NaOD buffer (100 mM) to generate dynamic system I. Considering that the imine formation process is pH-dependent, and that imines generally show higher stability under mildly basic conditions,²⁶⁻²⁷ the pH was set at a higher value. Bovine carbonic anhydrase is active within a wide pH range, from 4.5-9.5,²⁸ and pD 9.0 was therefore adopted for the screening process. ¹H NMR was used to monitor the constitutional changes with time in the presence or in the absence of CA for evaluation of the impacts of the enzyme on the constitutional distribution within the dynamic systems.

From the NMR spectra (Figure 2a-b), the CH_2NH_2 (n=1 or 2) resonances of compounds **2**, **3**, **4** and **5** could be clearly distinguished from each other, and used as characteristic peaks for quantification purposes. After two days at 40 °C, as witnessed from the NMR spectra, compound **5** was fully consumed, while the excess amines **3** and **4**, together with compound **2**, reached new equilibria. This indicates the progression of the imine exchange reaction, where distinct levels of compounds **3**, **4** and **5** were incorporated into the dyanmer main chain, whereas compound **2** dissociated from the original dyanmer **1-2**.

Initially, some peak overlapping was observed in the NMR spectra when comparing systems generated in the presence or absence of CA, likely due to H-bonding between the DCFs and CA. For this reason, a small amount of NaOD (final concentration of 8 nM) was added to both systems, instantly resulting in visible peak separations. Thus, compounds **2**, **3** and **4** could be clearly discerned, whereas no resonance of amine **5** was visible in either case. Using the signal of free compound **2** to represent the total formation of newly formed imine bonds, CA thus directed the incorporation of more amine **4** (39% compared to 30%) and less amine **3** (21% compared to 29%) upon re-equilibration. Meanwhile, the total conversions of the imine exchange reactions remained similar in both cases, calculated as 39% and 41% respectively. These results indicate that CA causes a significant change in the DCF structure, directing the demotion and promotion of optimal amines into the DCFs.

In order to amplify the effects of the enzyme selection preferences toward a given microenvironment, a few parameters were adjusted. Firstly, the molar ratio of each component **1-5** was changed from 1:1:1:0.5:0.5 to 1:0.75:1.5:0.75:0.75. As a result, the same trend was observed: the addition of CA provided more of free amine **4** (52% compared to 47%) and less amine **3** (20% compared to 24%), but the distribution differences were smaller. Moreover, the pD value of the buffer was also increased from 9.0 to 11.0 to maintain a clear solution throughout the whole screening process. At this higher pD, a higher consumption of free amine **4** (9% more) was observed in the absence of CA, leading to a larger distribution difference for the systems with or without CA.

The results for dynamic system I with respect to compound **5** indicated its total incorporation in the DCFs under either condition, and the preference of CA for this amine could not be evaluated. As a consequence, amine **5** was replaced by (S)-1-amino-

2-propanol (**6**) to generate dynamic system II (Figure 1, red). In this case, the addition of CA showed the same preferences (Figure 2c-d): more of free amine **4** (19% compared to 15%) and less amine **3** (12% compared to 14%). Meanwhile, the percentage of amine **2** and the total conversion of the imine exchange reaction were almost the same.

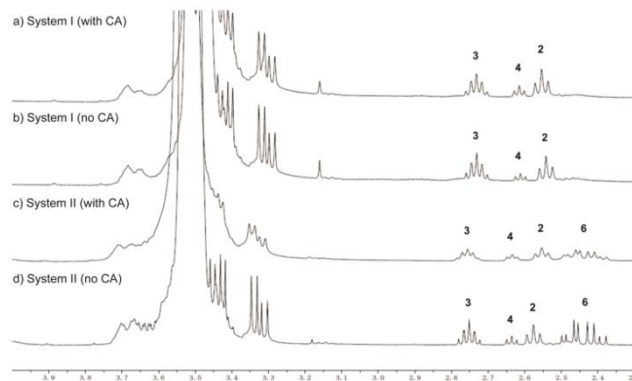


Figure 2. ¹H NMR spectra showing constitutional distribution changes in dynamic systems I and II in the presence or absence of CA.

To clearly demonstrate the constitutional distribution changes in dynamic system II, the conversion to imine DCF from each single amine **3**, **4** and **6** was calculated together with the differences obtained. As can be seen in Figure 3, in the presence of CA, the incorporation of amine **4** into the DCF was obviously inhibited (**1-2-4**), while the formation of DCFs **1-2-3** and **1-2-6** were promoted to some extent. Compared to DCF **1-2-3**, DCFs **1-2-4** and **1-2-6** showed lower conversion. Therefore, the influences of CA on the formation of different DCFs can be concluded as: **1-2-3** > **1-2-6** > **1-2-4**.

To connect the observed component preferences with the enzyme activation effects, DCFs **1-2-3**, **1-2-4** and **1-2-6** were synthesized individually. The activity of CA upon addition of each DCF was subsequently measured by colorimetric monitoring of the hydrolysis of p-nitrophenyl acetate (p-NPA). As seen in Figure 4, all DCFs increased the activity of the enzyme compared to when no additives were added. Interestingly, DCF **1-2-4** - whose formation was inhibited by CA in dynamic system II - possessed the strongest activation effect, increasing the enzyme activity to more than threefold its original value. On the other hand, DCF **1-2-6** also displayed a relatively good activation effect, leaving DCF **1-2-3** - the most favored DCF - the weakest activator of CA. This is likely a consequence of competition between the transimination reaction and the affinity of small amine compounds to CA. Since the CA protein structure holds the potential to form, e.g., H-bonds with the amines **3**, **4** and **6**, their reactivity towards transimination would be lowered. Thus, the higher the protein affinity of the amine, the lower the resulting availability to be incorporated into the DCFs. The dynamic frameworks may furthermore play the role of multivalent proton sponges that facilitate the shuttling of protons from the zinc-bound water molecules and the external medium, thereby accelerating the catalytic process.²⁹

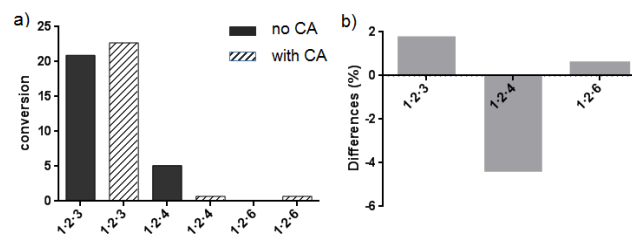


Figure 3. a) Constitutional distribution in dynamic system II (the conversions were calculated relative to exchanged/formed imino groups of starting dyanmer **1-2** to each indicated product); b) the calculated distribution differences (% conversion with CA - % conversion without CA).

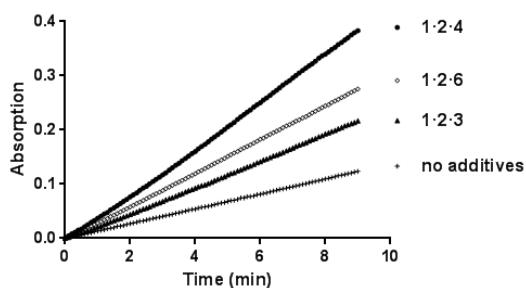


Figure 4. Activation effects of dynamers on catalytic activity of CA.

The amine monomers **3**, **4** and **6** were also tested for their influences on the CA activity (cf. **Figure S9a**). Although slightly higher catalytic activities were detected upon addition of the three amines, they effects were considerably lower than with the DCFs. These results indicate the importance of the DCF formation, which not only enabled potential multivalent effects, but also increased affinities to the enzyme, and enhanced activation effects. Moreover, the same activation trend ($4 > 6 > 3$) was observed for both the monomers and DCFs, demonstrating the contribution of amine **4** to a better microenvironment of CA, which is also consistent with the NMR results from the systemic screening process.

The changes of enzyme reaction kinetics upon the addition of the DCFs were next examined. The catalytic turnover number (k_{cat}) of the original enzyme reaction was determined as 0.64 $\mu\text{M}/\text{min}$, and the value was dramatically increased upon the addition of different DCFs. For example, in the presence of 2 mM of each DCF, the estimated k_{cat} values were 1.0 $\mu\text{M}/\text{min}$, 4.5 $\mu\text{M}/\text{min}$ and 1.6 $\mu\text{M}/\text{min}$ for DCF **1-2-3**, **1-2-4** and **1-2-6**, respectively. These results are consistent with the activation effects mentioned above.

To further explain the correlation between the enzyme selection preferences and the activation effects of the different DCFs, the CA-DCF interactions were monitored by fluorescence spectroscopy. Quenching of the CA emission band at 343 nm (Ex: 280 nm) was used to follow the binding events, from which results the apparent association constants (K_a) could be estimated. Thus, increasing amounts of DCFs **1-2-3**, **1-2-4** and **1-2-6** were introduced into CA solutions and the residual fluorescence recorded (cf. **Figure S10**). Based on the Stern–Volmer equation, the apparent K_a -values were estimated to range from 900–1200 M^{-1} for all the DCFs **1-2-3**, **1-2-4** and **1-2-6**. The similar affinities resulting from the different DCFs may partially be due to the binding of the poly(ethylene glycol) backbone to the enzyme.¹⁸ By comparison, the free amines **3**, **4** and **6** did not display enough binding strength towards CA, and the estimated apparent K_a -values were lower than 300 M^{-1} in each case.

The effects of the DCF on the enzyme were further studied by circular dichroism (CD) spectroscopy and titration experiments were carried out by adding increasing amount of each DCF to CA in buffer solutions. In the obtained CD-spectra (cf. **Figure S11a**), the intensities of the peak at around 210 nm decreased to some extent upon the first addition of each of the three DCFs, indicating slight changes in the enzyme's β -sheet structure due to the interactions between the DCFs and CA. Compared to DCF **1-2-6**, continuously increasing the concentration of DCF **1-2-4** however resulted in recovery of the peak intensity, showing a more favorably matched protein-DCF interaction. In contrast, when the concentration of DCF **1-2-3** increased to 0.75 mM, the

CD trace started to lose its shape in the range of 220–235 nm, probably coming from the slightly disordered CA structure. This distinct behavior of the three DCFs reflects the preference of CA for a specific surface microenvironment. In combination with the NMR- and UV-spectroscopy results, this demonstrates a clear correlation between the DCF composition and the protein interaction.

To further demonstrate the application potential of the screened DCFs, they were subsequently integrated into materials to immobilize the enzymes. For example, by mixing the modified DCF **1-2-4** solution (composition ratio **1:2:4** adjusted to 1:0.5:0.5) with water-soluble *O*-carboxymethyl chitosan in the presence of CA, hydrogels were formed. These CA-containing hydrogels showed similar or slightly higher (around 10%) activity than the free enzyme (**Figure S13a**). Furthermore, freeze-drying yielded highly cross-linked, sponge-like xerogels that could be repetitively used. However, further optimization of these initial gelation conditions are needed to better immobilize the enzymes and prevent leaching (**Figure S13b**).

In conclusion, we have established a dynamic optimization system based on the enzyme-directed identification of the most favorable microenvironment with respect to enzyme activity. Constitutional dynamic systems were generated from imine-linked polymers (**1-2**) and different amines (**3-6**), to which carbonic anhydrase was added. The interactions between the DCFs and protein surface led to different component distributions in the systems compared to when CA was absent. Evaluation of the enzyme activation effects resulted in a correlation between the perturbation of the dynamic system and the activity. An optimal DCF composition proved important, in this case suggesting that amines with lower enzyme-directed incorporation ratio possess higher enzyme activation effects, where DCF **1-2-4** turned out to be the best activator. The enzyme kinetic studies were in good correlation with the activation effects of the DCFs, providing insights into the CA-substrate interactions upon addition of the DCFs.

Furthermore, fluorescence spectroscopy was used to confirm and quantitate the CA-DCF interactions, while CD spectroscopy was adopted to study the enzyme structural changes upon addition of the DCFs. The obtained results showed smaller differences among DCFs **1-2-3**, **1-2-4** and **1-2-6** compared to their performances in dynamic screening process, indicating the amplification effect from the constitutional dynamic systems, which were carried out in a one-pot competitive mode. This dynamic structural exchange, leading to adaptive enzyme encapsulation/binding (“DCFic effect”) and potentially coupled to a “proton sponge” effect, was directly affecting the enzyme activation.¹⁸⁻²⁰ Moreover, the selected DCFs could be applied in enzyme immobilization protocols by integration into hydrogels. Thus, the model system established here demonstrates the first case of protein surface-directed dynamic screening for optimization of the catalytic microenvironment.

The combined features of new systemic strategy - enhanced enzyme catalytic performances - potential addressability, make processes and species such as those presented here of interest for the development of clinical application for the treatment of a multitude diseases or environmental application for efficient carbon capture systems

ASSOCIATED CONTENT

Supporting Information.

The supporting information including NMR, UV, fluorescent and CD spectra is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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REFERENCES

1. Lehn, J.-M., From supramolecular chemistry towards constitutional dynamic chemistry and adaptive chemistry. *Chem. Soc. Rev.* **2007**, *36* (2), 151-160.
2. Lehn, J.-M., Perspectives in chemistry—aspects of adaptive chemistry and materials. *Angew. Chem., Int. Ed.* **2015**, *54* (11), 3276-3289.
3. Frei, P.; Hevey, R.; Ernst, B., Dynamic combinatorial chemistry: a new methodology comes of age. *Chem. Eur. J.* **2019**, *25* (1), 60-73.
4. Reuther, J. F.; Dees, J. L.; Kolesnichenko, I. V.; Hernandez, E. T.; Ukraintsev, D. V.; Guduru, R.; Whiteley, M.; Anslyn, E. V., Dynamic covalent chemistry enables formation of antimicrobial peptide quaternary assemblies in a completely abiotic manner. *Nat. Chem.* **2017**, *10*, 45.
5. Jin, Y.; Wang, Q.; Taynton, P.; Zhang, W., Dynamic covalent chemistry approaches toward macrocycles, molecular cages, and polymers. *Acc. Chem. Res.* **2014**, *47* (5), 1575-1586.
6. Schaufelberger, F.; Ramström, O., Kinetic self-sorting of dynamic covalent catalysts with systemic feedback regulation. *J. Am. Chem. Soc.* **2016**, *138* (25), 7836-7839.
7. Schaufelberger, F.; Ramström, O., Dynamic covalent organocatalysts discovered from catalytic systems through rapid deconvolution screening. *Chem. Eur. J.* **2015**, *21* (36), 12735-12740.
8. Zhou, Y.; Li, C.; Peng, J.; Xie, L.; Meng, L.; Li, Q.; Zhang, J.; Li, X. D.; Li, X.; Huang, X.; Li, X., DNA-encoded dynamic chemical library and its applications in ligand discovery. *J. Am. Chem. Soc.* **2018**, *140* (46), 15859-15867.
9. Hauke, T. J.; Wein, T.; Höfner, G.; Wanner, K. T., Novel allosteric ligands of γ -aminobutyric acid transporter 1 (GAT1) by MS based screening of pseudostatic hydrazone libraries. *J. Med. Chem.* **2018**, *61* (22), 10310-10332.
10. Wei, Z.; Yang, J. H.; Zhou, J.; Xu, F.; Zrínyi, M.; Dussault, P. H.; Osada, Y.; Chen, Y. M., Self-healing gels based on constitutional dynamic chemistry and their potential applications. *Chem. Soc. Rev.* **2014**, *43* (23), 8114-8131.
11. Liu, Y.; Lehn, J.-M.; Hirsch, A. K. H., Molecular biodynamers: dynamic covalent analogues of biopolymers. *Acc. Chem. Res.* **2017**, *50* (2), 376-386.
12. Zhang, Y.; Jayawardena, H. S. N.; Yan, M.; Ramström, O., Enzyme classification using complex dynamic hemithioacetal systems. *Chem. Commun.* **2016**, *52* (28), 5053-5056.
13. Jumde, V. R.; Mondal, M.; Gierse, R. M.; Unver, M. Y.; Magari, F.; van Lier, R. C. W.; Heine, A.; Klebe, G.; Hirsch, A. K. H., Design and synthesis of bioisosteres of acylhydrazones as stable inhibitors of the aspartic protease endothiapepsin. *ChemMedChem* **2018**, *13* (21), 2266-2270.
14. Zhang, Y.; Hu, L.; Ramström, O., Double parallel dynamic resolution through lipase-catalyzed asymmetric transformation. *Chem. Commun.* **2013**, *49* (18), 1805-1807.
15. Zhang, Y.; Vongvilai, P.; Sakulsombat, M.; Fischer, A.; Ramström, O., Asymmetric synthesis of substituted thiolanes through domino thia-Michael-Henry dynamic covalent systemic resolution using lipase catalysis. *Adv. Synth. Catal.* **2014**, *356* (5), 987-992.
16. Hu, L.; Schaufelberger, F.; Zhang, Y.; Ramström, O., Efficient asymmetric synthesis of lamivudine via enzymatic dynamic kinetic resolution. *Chem. Commun.* **2013**, *49* (88), 10376-10378.
17. Bilal, M.; Cui, J.; Iqbal, H. M. N., Tailoring enzyme microenvironment: State-of-the-art strategy to fulfill the quest for efficient bio-catalysis. *Int. J. Biol. Macromol.* **2019**, *130*, 186-196.
18. Zhang, Y.; Legrand, Y.-M.; Petit, E.; Supuran, C. T.; Barboiu, M., Dynamic encapsulation and activation of carbonic anhydrase in multivalent dimeric host matrices. *Chem. Commun.* **2016**, *52* (21), 4053-4055.
19. Zhang, Y.; Feng, W.-X.; Legrand, Y.-M.; Supuran, C. T.; Su, C.-Y.; Barboiu, M., Dynameric host frameworks for the activation of lipase through H-bond and interfacial encapsulation. *Chem. Commun.* **2016**, *52* (95), 13768-13770.
20. Zhang, Y.; Supuran, C. T.; Barboiu, M., Exponential activation of carbonic anhydrase by encapsulation in dynameric host matrices with chiral discrimination. *Chem. Eur. J.* **2018**, *24* (3), 715-720.
21. Supuran, C. T., Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opin. Drug Discovery* **2017**, *12* (1), 61-88.
22. Huc, I.; Lehn, J.-M., Virtual combinatorial libraries: Dynamic generation of molecular and supramolecular diversity by self-assembly. *Proc. Natl. Acad. Sci.* **1997**, *94* (6), 2106-2110.
23. Nasr, G.; Petit, E.; Vullo, D.; Winum, J.-Y.; Supuran, C. T.; Barboiu, M., Carbonic Anhydrase-Encoded Dynamic Constitutional Libraries: Toward the Discovery of Isozyme-Specific Inhibitors. *J. Med. Chem.* **2009**, *52* (15), 4853-4859.
24. Supuran, C. T., Carbonic anhydrase activators. *Future Med. Chem.* **2018**, *10* (5), 561-573.
25. Licsandru, E.; Tanc, M.; Kocsis, I.; Barboiu, M.; Supuran, C. T., A class of carbonic anhydrase I – selective activators. *J. Enzyme Inhib. Med. Chem.* **2017**, *32* (1), 37-46.
26. Scheller, P. N.; Lenz, M.; Hammer, S. C.; Hauer, B.; Nestl, B. M., Imine reductase-catalyzed intermolecular reductive amination of aldehydes and ketones. *ChemCatChem* **2015**, *7* (20), 3239-3242.
27. Belowich, M. E.; Stoddart, J. F., Dynamic imine chemistry. *Chem. Soc. Rev.* **2012**, *41* (6), 2003-2024.
28. Demir, Y.; Demir, N.; Nadaroglu, H.; Bakan, E., Purification and characterization of carbonic anhydrase from bovine erythrocyte plasma membrane. *Prep. Biochem. Biotechnol.* **2000**, *30* (1), 49-59.
29. Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T., Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry* **1997**, *36* (34), 10384-10392.

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