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Artificial amidase with modifiable active sites and designable substrate selectivity for aryl amide hydrolysis†

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Hydrolases are used by cells to process key biomolecules including peptides and esters. Previous synthetic mimics of proteases generally only hydrolyze highly active ester derivatives. We report a synthetic catalyst with an acid/base dyad in its active site that hydrolyzes aryl amides under near physiological conditions. The aspartic protease mimic achieves substrate selectivity by its imprinted active site, which is tunable through different template molecules used during molecular imprinting. It can be designed to maintain or override the intrinsic activities of aryl amides in a predictable manner.

Hydrolases are vital for cells to process key biomolecules including peptides, lipids, nucleic acids, and carbohydrates. Spontaneous hydrolyses of amide, phosphate ester, or glycosidic bonds take hundreds to tens of millions of years.¹ Chemists can speed up these reactions by activating the electrophile (*e.g.*, protonation of carbonyl), the nucleophile (*e.g.*, deprotonation of water), and/or the leaving group (*e.g.*, by protonation of a glycosidic oxygen). Enzymes employ the same fundamental strategies to speed up their catalytic reactions,² but they do so inside their active sites instead of changing the entire reaction media as chemists do. This allows enzymes to target specific substrates (or specific sites on a substrate) in the presence of similar or more reactive functional groups in the same solution—a selectivity difficult to realize with synthetic catalysts.

Aspartic proteases employ a pair of carboxylic acids for catalytic amide hydrolysis.³ One of the two acids is deprotonated and the resulting carboxylate/carboxylic acid dyad is able to activate the electrophilic substrate, nucleophilic water, and the amine leaving group cooperatively.⁴

In this work, we set our goal to employ an acid/base dyad to duplicate the function of aspartic protease inside a

synthetically constructed active site. Many efforts have been made to prepare protease-mimicking synthetic catalysts, but they generally only hydrolyze highly activated esters.^{5–19} Limited success has been reported in creating artificial enzymes to hydrolyze amides, *i.e.*, artificial amidase.^{20–22} Natural enzymes, for example, use their active sites to distinguish closely related substrates and are even able to overturn intrinsic reactivities of the substrates. These features are yet to be realized in the synthetic mimics of protease.

To create our artificial amidase, we first designed template molecule **1a**, whose structure is color-coded to highlight the different purposes of the substructures (Scheme 1): the red moiety is the space holder for the substrate and the blue part indicates the to-be-installed catalytic dyad; and the black part allows the template to be polymerized into a cross-linked polymer network.

To create enzyme-like water-soluble nanoparticles, we performed molecular imprinting^{23–32} of **1a** in the micelle of cross-linkable surfactant **2**. The mixed micelle, containing divinyl benzene (DVB, a free radical cross-linker) and 2,2-dimethoxy-2-phenylacetophenone (DMPA, a photo initiator), is first cross-linked on the surface by diazide **3** *via* the highly efficient click reaction (Scheme 1, step a). A second round of click reaction with monoazide **4** introduces a layer of hydrophilic ligands on the micelle surface (step b). In step c, UV-induced photopolymerization co-polymerizes the template with polymerizable surfactant **2** and DVB, solidifying the micelle core to afford nanoparticles, NP(**1a**).

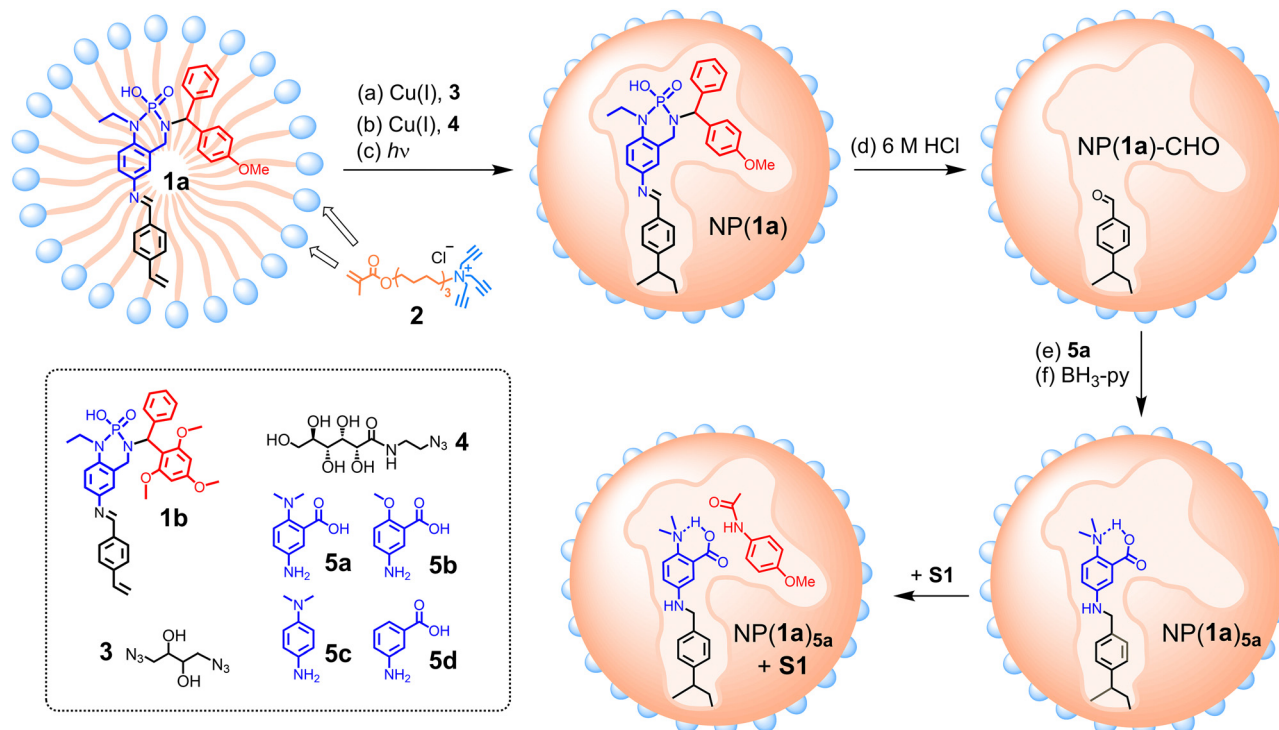
In the post-modification, the imine bond of the polymerized template inside NP(**1a**) is first hydrolyzed in 6 M HCl to afford an aldehyde group in NP(**1a**)-CHO (step d), which is functionalized through reductive amination with **5a** (steps e and f). The resulting NP(**1a**)_{5a}, *i.e.*, the imprinted nanoparticle prepared with **1a** as the template and post-modified with **5a** in the reductive amination, contains an acid/base pair in the active site, with a nearby substrate-binding site similar in size and shape to the red-colored moiety of the template. As shown in Scheme 1, this site is designed to accommodate 4-methoxyphenyl acetanilide (**S1**), with the carbonyl close to the catalytic dyad.

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Scheme 1 Preparation of molecularly imprinted nanoparticles NP(1a)_{5a} as artificial amidase for the hydrolysis of **S1**. Surface ligands (*i.e.*, clicked **4**) on the micelle surface are omitted for clarity.

NP(1a)_{5a} indeed is able to perform catalytic hydrolysis of aryl amides (**S1** and **S2**), but not less reactive amides such as **S3** and **S4** (Fig. 1a). Note that, once taken out of the active site, **5a** is completely inactive toward either 4-nitrophenyl acetate (an activated ester) or 4-nitrophenyl acetanilide (**S2**) (Fig. S3–S6, ESI†). Substrate **S2** has a better leaving group than **S1**, but only affords *ca.* 1/3 of the hydrolytic product in the NP(1a)_{5a}-catalyzed reaction (Fig. 1a). The overturn of intrinsic reactivity is a strong indicator for successful molecular imprinting. After all, template **1a** with its *para*-methoxyphenyl group is designed to bind **S1** that contains the same substructure. Molecularly imprinted micelles have been shown to have outstanding abilities to distinguish small changes in their guests during binding, including the addition,³³ removal,³³ and shift³⁴ of a single methyl (or methylene) group. Covalent imprinting, as in

our case, is also known to have high fidelity in the imprinting process.^{35,36}

Since the imprinted active site strongly influences the substrate selectivity, we prepared NP(1b)_{5a} using template **1b** that is expected to afford a larger and more accommodating substrate-binding site. Both **S1** and **S2** become equally reactive with this catalyst under the experimental conditions (20 h at 80 °C in pH 8 buffer) while **S3** and **S4** stay unreactive (Fig. 1a). It seems that the larger imprinted pocket can now reasonably accommodate the *para*-nitro-substituted substrate, and the higher intrinsic reactivity allows **S2** to catch up with **S1** (see below for additional discussion).

Fig. 1b shows the pH profile for the hydrolysis of **S2** by NP(1b)_{5a}. A large change in catalytic activity happens over pH 6–8 while a further increase of pH brings a less significant effect. The same figure indicates that the aryl amide has negligible reactivity in the presence of the nonimprinted nanoparticles (NINPs, similarly prepared nanoparticles in the absence of the template) or in the buffer. To further confirm the importance of the imprinted pocket and the catalytic dyad, we studied the hydrolysis of **S2** by several other control catalysts, *i.e.*, NP(1b)-CHO or NP(1b) with an aldehyde group in the imprinted site after the imine hydrolysis, and NP(1b)_{5b–d} (*i.e.*, the nanoparticles obtained through reductive amination of NP(1b)-CHO using compounds **5b–d**). The fact that none of these nanoparticles display significant activities (Fig. S19, ESI†) indicates that both the amine and the carboxylic acid in NP(1b)_{5a} are critical to the observed activity.

NP(1b)_{5a} exhibits enzyme-like Michaelis–Menten kinetics in its catalytic hydrolysis of **S2** under near physiological conditions

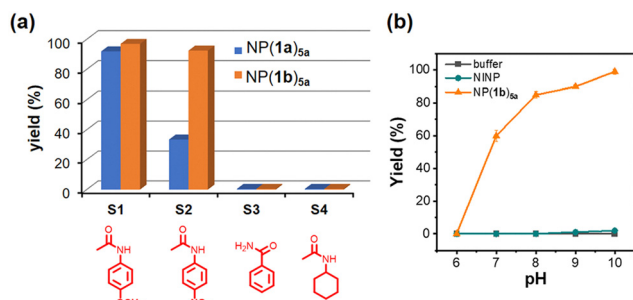


Fig. 1 (a) Yields of amide hydrolyses by NP(1a)_{5a} and NP(1b)_{5a} after 20 h at 80 °C in pH 8 buffer. [substrate] = 50 μM. [catalyst] = 10 μM. (b) The pH profile in the hydrolysis of **S2** by NP(1b)_{5a}.

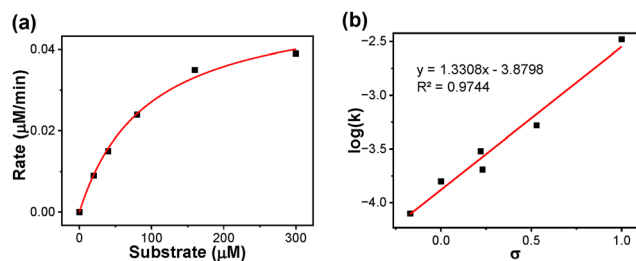


Fig. 2 (a) Michaelis-Menten plot of the hydrolysis of **S2** by NP(**1b**)_{5a} in a 25 mM HEPES buffer at 40 °C and pH 7.4. [NP(**1b**)_{5a}] = 8 μM. (b) Hammett σ - ρ correlation in the hydrolysis of *para*-substituted-phenyl acetates catalyzed by NP(**1b**)_{5a}.

(Fig. 2a). With a Michaelis constant (K_m) of 94 ± 11 μM and the catalytic turnover (k_{cat}) of 0.0065 min^{-1} , the catalytic efficiency (k_{cat}/K_m) is $69 \text{ M}^{-1} \text{ min}^{-1}$. Natural enzymes tend to have high turnovers but moderate substrate binding. In contrast, our catalysis is dominated by substrate binding, likely because the template bears too close a resemblance to the substrate instead of the transition state.

We probed the catalytic mechanism of NP(**1b**)_{5a} by two methods, employing more reactive *p*-substituted phenyl acetates as commonly done in both natural^{37,38} and artificial enzymes.^{5–19} Hammett plots reveal the amount of negative charge developed on the phenyl oxygen in aryl ester hydrolysis. Anionic oxygen-based nucleophiles tend to give a reaction constant (ρ) of 1–1.2.^{39–41} In contrast, nucleophilic attack of the ester by a neutral nitrogen affords a ρ of 2–3, while a general-base-catalyzed water attack has a ρ of 0.5–0.7. The ρ value of NP(**1b**)_{5a} for aryl ester hydrolysis is 1.33 (Fig. 2b), consistent with an anionic nucleophile in the active site, likely the carboxylate after intramolecular proton transfer to the *ortho* amine (Scheme 1).

The second mechanistic investigation involves the solvent kinetic isotope effect (KIE): reactions with a cleavage of the O–H bond in the rate-determining step such as a general-base catalysis has a primary KIE of $k_{H_2O}/k_{D_2O} = 2$ –3.^{41–43} In our case, a KIE value of 1.06 is obtained for the hydrolysis of *p*-nitrophenyl acetate by NP(**1b**)_{5a} (Table S1, ESI†), ruling out such mechanisms. Apparently, the proton transfer from the carboxylic acid to the neighboring amine occurred prior to the rate-determining step, allowing the carboxylate to carry out the nucleophilic attack as an anionic oxygen nucleophile, also supported by the Hammett plot as discussed above.

It should be mentioned that nucleophilic attack on the carbonyl is just one of the many steps in the amide hydrolysis, which involves a much poorer leaving group than the aryl esters. Departure of the leaving group and hydrolysis of the acylated catalyst need to occur before the catalyst is ready for another round of catalysis. The pH profile shown in Fig. 1b should be a composite effect, as all these steps are pH-dependent.

Why is the same dyad highly active inside the molecularly imprinted site but completely inactive in solution? A key reason is probably proximity, since the substrate is bound by the

imprinted site, with its carbonyl right next to the catalytic groups. Desolvation likely is also highly important. The carboxylate can hydrogen-bond easily with solvent molecules in water and become less nucleophilic. This explains why **5a** is inactive in methanol or water. Once the dyad resides in a hydrophobic pocket, not only does the lack of nearby protic solvent molecules increase the nucleophilicity of the carboxylate, but the *ortho* amine after turning into the ammonium ion (*i.e.*, the conjugate acid) can also stabilize the anionic tetrahedral intermediate. Electrostatic interactions are especially strong in a nonpolar microenvironment and have been proposed to be a major contributor to enzyme catalysis.⁴⁴

Fig. 1a shows that NP(**1b**)_{5a} with its larger substrate-binding site is less selective than NP(**1a**)_{5a}. It nonetheless is able to differentiate the substitution pattern on the phenyl group of aryl amides. As shown in Fig. 3, it tolerates substrates with various *para* groups on the phenyl ring (**S1**, **S2**, **S5**, and **S6**) and also *ortho*-nitrophenyl acetamide (**S7**). Consistent with the 2,4,6-trimethoxy substitution on its template (**1b**), despite its broad substrate selectivity, NP(**1b**)_{5a} completely excludes *meta*-nitrophenyl acetamide (**S8**).

S1 is more reactive than **S2** with NP(**1a**)_{5a} but the two become similarly reactive in the presence of NP(**1b**)_{5a} (Fig. 1a). Under a milder condition (at 40 °C and pH 7.4), **S2** overtakes **S1**. It seems that when competing factors are at play (*e.g.*, intrinsic reactivity *versus* templating effect), the final selectivity becomes a tradeoff and can vary under different reaction conditions.

This work shows that a simple acid/base catalytic dyad inside a substrate-tailored imprinted pocket can hydrolyze aryl amides under near physiological conditions (pH 7.4 at 40 °C) but the same dyad is completely unreactive in solution. A highlight of the system is the readily tunable substrate-binding site, *via* different template molecules (**1a** and **1b**). Tunable reactivity is then achieved, a feature commonly seen in natural enzymes but rare in synthetic systems.^{45,46} As a result, aryl amides with less intrinsic reactivity can be made more reactive and substitution patterns on the substrate are easily distinguished in a predictable manner.

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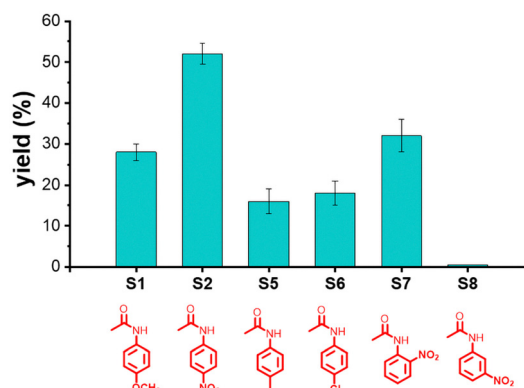


Fig. 3 The yields of aryl amide hydrolysis catalyzed by NP(**1b**)_{5a} after 24 h in HEPES buffer (pH 7.4) at 40 °C.



Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

Notes and references

- 1 R. Wolfenden, *Annu. Rev. Biochem.*, 2011, **80**, 645–667.
- 2 E. Erez, D. Fass and E. Bibi, *Nature*, 2009, **459**, 371–378.
- 3 B. M. Dunn, *Chem. Rev.*, 2002, **102**, 4431–4458.
- 4 D. B. Northrop, *Acc. Chem. Res.*, 2001, **34**, 790–797.
- 5 D. J. Cram, H. E. Katz and I. B. Dicker, *J. Am. Chem. Soc.*, 1984, **106**, 4987–5000.
- 6 V. T. D'Souza and M. L. Bender, *Acc. Chem. Res.*, 1987, **20**, 146–152.
- 7 K. Skorey, V. Somayaji and R. Brown, *J. Am. Chem. Soc.*, 1989, **111**, 1445–1452.
- 8 T. Ema, D. Tanida, T. Matsukawa and T. Sakai, *Chem. Commun.*, 2008, 957–959.
- 9 H. B. Albada and R. M. J. Liskamp, *J. Comb. Chem.*, 2008, **10**, 814–824.
- 10 M. Kheirabadi, N. Çelebi-Ölçüm, M. F. L. Parker, Q. Zhao, G. Kiss, K. N. Houk and C. E. Schafmeister, *J. Am. Chem. Soc.*, 2012, **134**, 18345–18353.
- 11 M. J. MacDonald, L. D. Lavis, D. Hilvert and S. H. Gellman, *Org. Lett.*, 2016, **18**, 3518–3521.
- 12 J. J. Garrido-González, M. M. Iglesias Aparicio, M. M. García, L. Simón, F. Sanz, J. R. Morán and Á. L. Fuentes de Arriba, *ACS Catal.*, 2020, **10**, 11162–11170.
- 13 J. J. Garrido-González, E. Sánchez-Santos, A. Habib, Á. V. Cuevas Ferreras, F. Sanz, J. R. Morán and Á. L. Fuentes de Arriba, *Angew. Chem., Int. Ed.*, 2022, **61**, e202206072.
- 14 P. Pengo, S. Polizzi, L. Pasquato and P. Scrimin, *J. Am. Chem. Soc.*, 2005, **127**, 1616–1617.
- 15 M. D. Nothling, A. Ganesan, K. Condic-Jurkic, E. Pressly, A. Davalos, M. R. Gotrik, Z. Xiao, E. Khoshdel, C. J. Hawker, M. L. O'Mara, M. L. Coote and L. A. Connal, *Chem*, 2017, **2**, 732–745.
- 16 M. D. Nothling, Z. Xiao, N. S. Hill, M. T. Blyth, A. Bhaskaran, M.-A. Sani, A. Espinosa-Gomez, K. Ngov, J. White, T. Buscher, F. Separovic, M. L. O'Mara, M. L. Coote and L. A. Connal, *Sci. Adv.*, 2020, **6**, eaaz0404.
- 17 I. Bose and Y. Zhao, *ACS Catal.*, 2021, **11**, 3938–3942.
- 18 N. Liu, S.-B. Li, Y.-Z. Zheng, S.-Y. Xu and J.-S. Shen, *ACS Omega*, 2023, **8**, 2491–2500.
- 19 Z. Wang, Y. Lu, J. Yang, W. Xiao, T. Chen, C. Yi and Z. Xu, *Langmuir*, 2023, **39**, 5929–5935.
- 20 Y.-M. Wong, Y. Hoshino, K. Sudesh, Y. Miura and K. Numata, *Biomacromolecules*, 2015, **16**, 411–421.
- 21 M. Divya, T. Benny, P. Christy, E. P. Aparna and K. S. Devaky, *Bioorg. Chem.*, 2017, **74**, 91–103.
- 22 T. Matsushita, H. Yamochi, S. Omiya, T. Koyama, K. Hatano and K. Matsuoka, *Bioorg. Med. Chem.*, 2023, **92**, 117422.
- 23 G. Wulff and J. Liu, *Acc. Chem. Res.*, 2012, **45**, 239–247.
- 24 X. Shen, C. Huang, S. Shinde, K. K. Jagadeesan, S. Ekström, E. Fritz and B. Sellergrén, *ACS Appl. Mater. Interfaces*, 2016, **8**, 30484–30491.
- 25 Y. Yuan, Y. Yang, M. Faheem, X. Zou, X. Ma, Z. Wang, Q. Meng, L. Wang, S. Zhao and G. Zhu, *Adv. Mater.*, 2018, **30**, 1800069.
- 26 J. Pan, W. Chen, Y. Ma and G. Pan, *Chem. Soc. Rev.*, 2018, **47**, 5574–5587.
- 27 S. Li, P. A. Lieberzeit, S. Piletsky and A. P. F. Turner, *Smart polymer catalysts and tunable catalysis*, Elsevier, Amsterdam, Netherlands; Cambridge, MA, 2019.
- 28 H. Zhang, *Adv. Mater.*, 2020, **32**, 1806328.
- 29 K. Haupt, P. X. Medina Rangel and B. T. S. Bui, *Chem. Rev.*, 2020, **120**, 9554–9582.
- 30 S. Muratsugu, S. Shirai and M. Tada, *Tetrahedron Lett.*, 2020, **61**, 151603.
- 31 J. Li, M. Zhu, M. Wang, W. Qi, R. Su and Z. He, *Soft Matter*, 2020, **16**, 7033–7039.
- 32 W. Wei, V. K. Thakur, Y. J. Chew and S. Li, *Mater. Today Chem.*, 2020, **17**, 100286.
- 33 K. Chen and Y. Zhao, *Org. Biomol. Chem.*, 2019, **17**, 8611–8617.
- 34 J. K. Awino, R. W. Gunasekara and Y. Zhao, *J. Am. Chem. Soc.*, 2017, **139**, 2188–2191.
- 35 G. Wulff, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1812–1832.
- 36 G. Wulff, *Chem. Rev.*, 2002, **102**, 1–28.
- 37 J. A. Verpoorte, S. Mehta and J. T. Edsall, *J. Biol. Chem.*, 1967, **242**, 4221–4229.
- 38 F. J. Kezdy and M. L. Bender, *Biochemistry*, 1962, **1**, 1097–1106.
- 39 T. C. Bruice and S. J. Benkovic, *J. Am. Chem. Soc.*, 1964, **86**, 418–426.
- 40 J. F. Kirsch, W. Clewell and A. Simon, *J. Org. Chem.*, 1968, **33**, 127–132.
- 41 R. A. Gibbs, P. A. Benkovic, K. D. Janda, R. A. Lerner and S. J. Benkovic, *J. Am. Chem. Soc.*, 1992, **114**, 3528–3534.
- 42 M. L. Bender, E. J. Pollock and M. C. Neveu, *J. Am. Chem. Soc.*, 1962, **84**, 595–599.
- 43 B. M. Anderson, E. H. Cordes and W. P. Jencks, *J. Biol. Chem.*, 1961, **236**, 455–463.
- 44 A. Warshel, P. K. Sharma, M. Kato, Y. Xiang, H. B. Liu and M. H. M. Olsson, *Chem. Rev.*, 2006, **106**, 3210–3235.
- 45 M. D. Arifuzzaman, I. Bose, F. Bahrami and Y. Zhao, *Chem. Catal.*, 2022, **2**, 2049–2065.
- 46 F. Bahrami and Y. Zhao, *J. Org. Chem.*, 2024, **89**, 5148–5152.

