

An expanded dinuclear-zinc centre enables H_3O_2^- -mediated enzymatic aromatic substitution

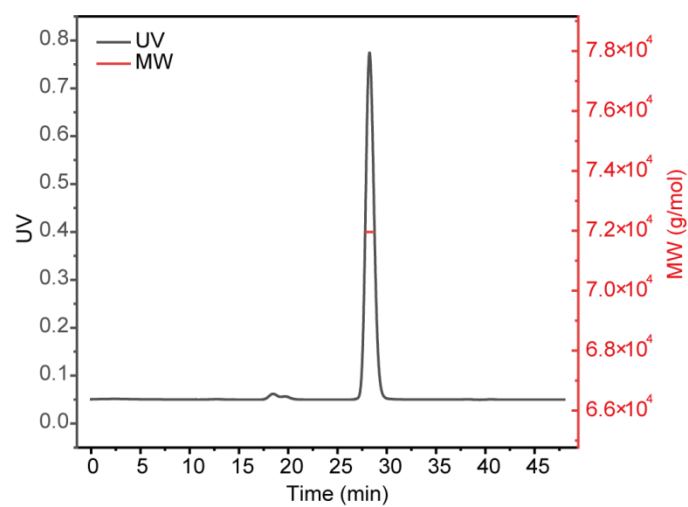
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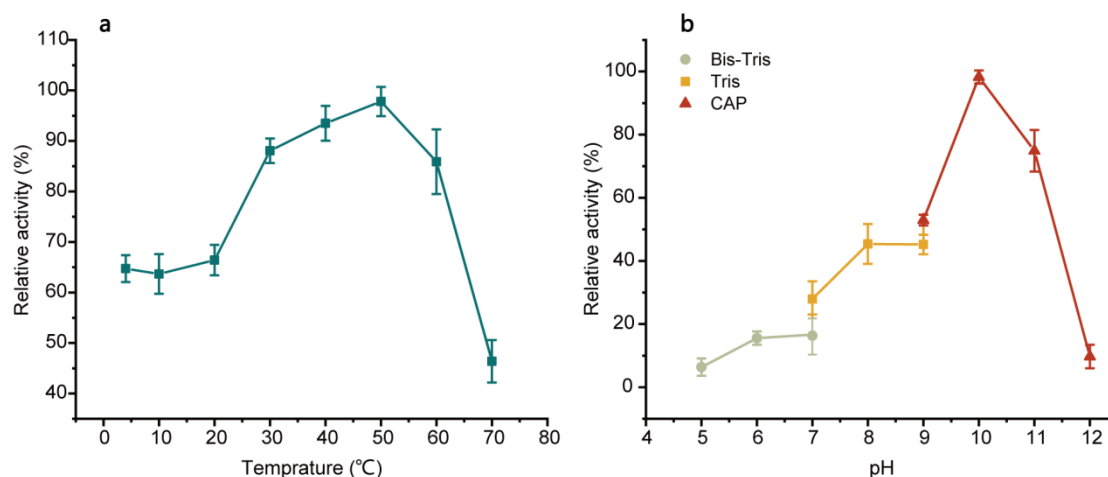
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Supplementary figure



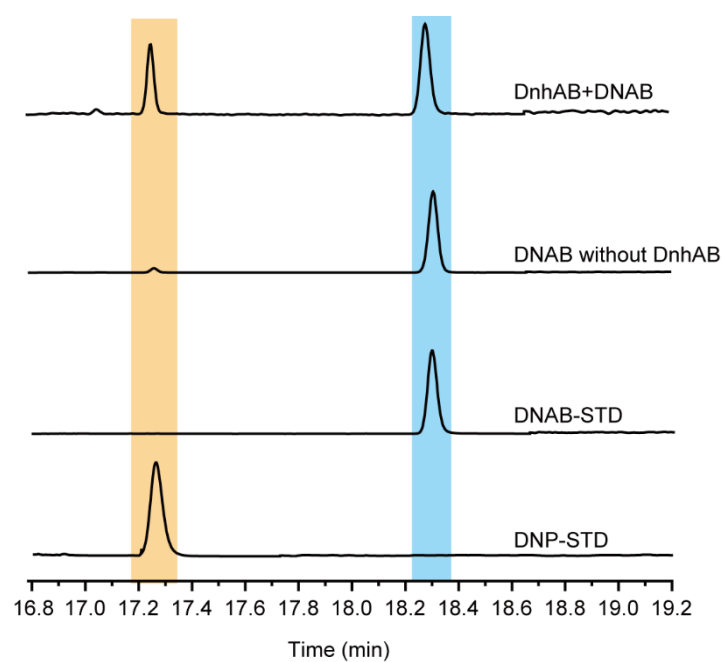
Supplementary Fig. 1 SEC-MALS analysis of DnhB. The molecular weight is determined to be $(7.196 \times 10^4 \pm 0.317\%)$ g/mol, indicating that DnhB exists as a dimer in solution.

Supplementary Information



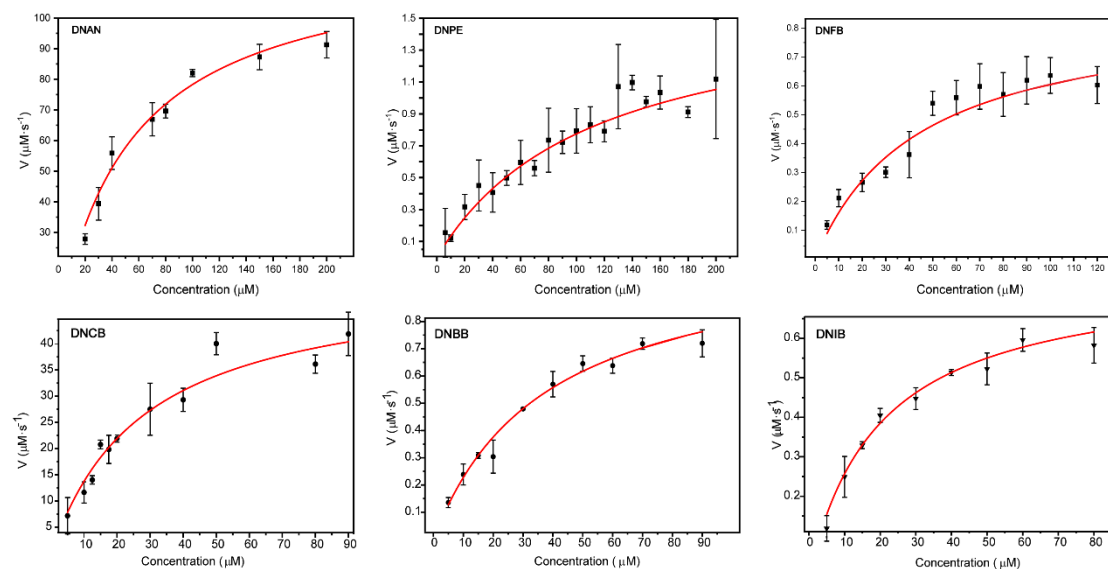
Supplementary Fig. 2 Optimization of reaction conditions for DnhAB. **a**, Temperature dependence of DnhAB activity. Enzyme activity was assayed in 50 mM Tris-HCl buffer (pH 8.0) supplemented with 2 nM DnhAB, and 10 mM DNAN was used to initiate the reaction across a temperature range of 5-70°C. The optimal reaction temperature is 50°C. **b**, The optimal pH was determined at 25°C using the following buffers: 20 mM Bis-Tris (pH 5-7), 20 mM Tris-HCl (pH 7-9), and 20 mM glycine-NaOH (pH 9-12). Error bars represent the standard deviation of three biological replicates. Source Data are provided as a Source Data file.

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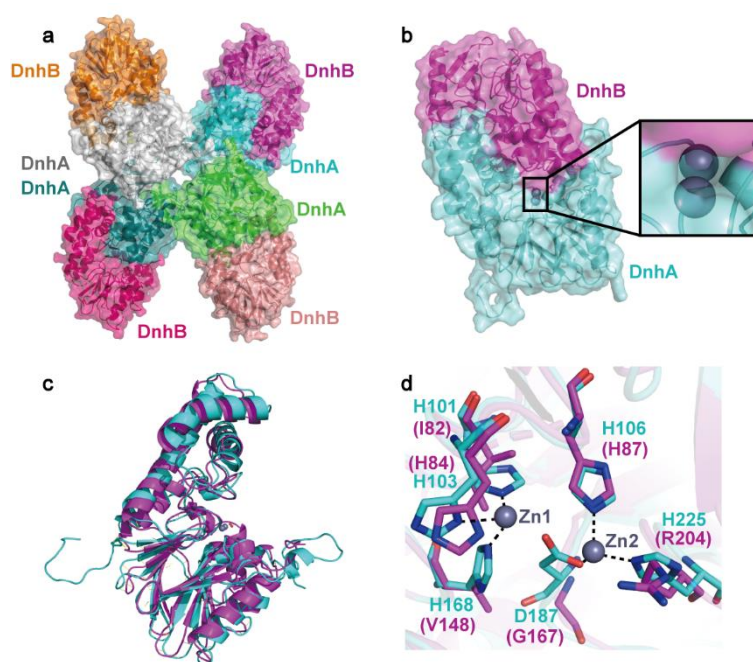


Supplementary Fig. 3 DnhAB catalyzes the hydrolysis of DNAB to DNP. Reactions were carried out in 50 mM Tris-HCl, pH 8.0, and analyzed by HPLC. No product (DNP) was detected in the control without the enzyme.

Supplementary Information

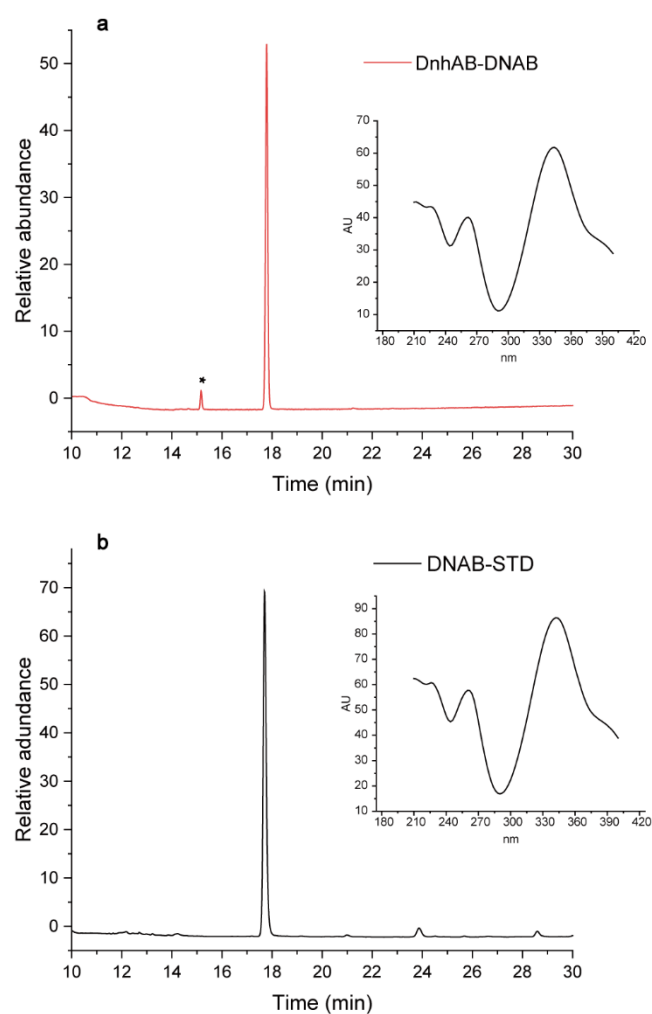


Supplementary Fig. 4 Kinetic characterization of DnhAB against different substrates. Michaelis–Menten plots for the DnhAB-catalyzed SNAr reaction with DNAN, DNPE, and halogenated nitroaromatic hydrocarbons. Enzyme activity was assayed in 50 mM Tris-HCl buffer (pH 8.0) supplemented with 2 nM DnhAB and an appropriate amount of substrate. The plots display averaged initial rates fitted to the Michaelis–Menten equation using Origin software. Error bars represent the standard deviation of triplicate measurements. Source data are provided as a Source Data file.



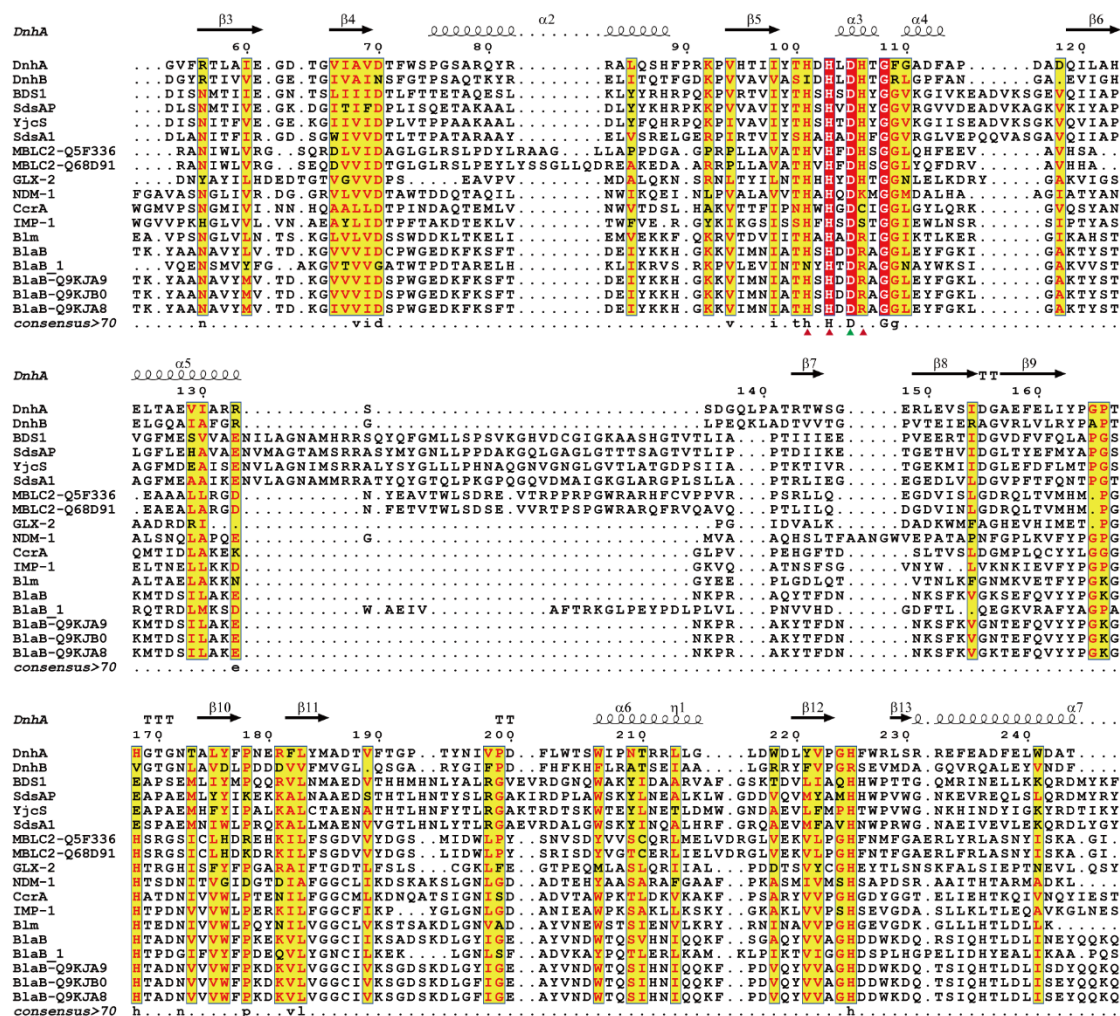
Supplementary Fig. 5 Structural features of DnhAB. **a** DnhAB forms an “X-shaped” hetero-octameric structure; **b** the active pocket is located in the cavity at the interface between subunits A and B; **c** superposition of DnhA and DnhB shows that both possess the $\alpha\beta\alpha$ scaffold characteristic of metallo- β -lactamases ($C\alpha$ RMSD $\approx 1.43\text{\AA}$); **d** the metal-binding sites of DnhA and DnhB overlap, but the corresponding residues in DnhB are mutated, rendering it unable to bind Zn(II).

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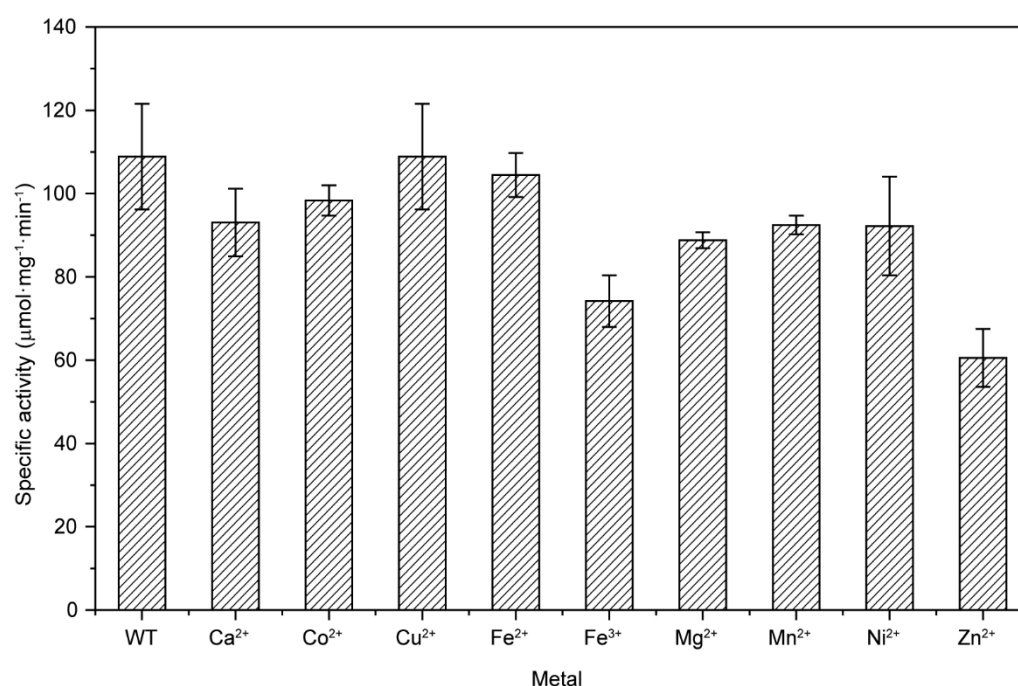


Supplementary Fig. 6 Liquid-phase analysis of the crystallization solution of the DnhAB-DNAB complex. After briefly soaking DnhAB crystals in 5 mM DNAB for 1 min, no significant formation of the product DNP was detected.

Supplementary Fig. 7 Multiple sequence alignment of the DnhAB active site (residues 50–248) and homologous regions. Red triangles mark metal-binding sites; green triangles indicate the proton donor D105.

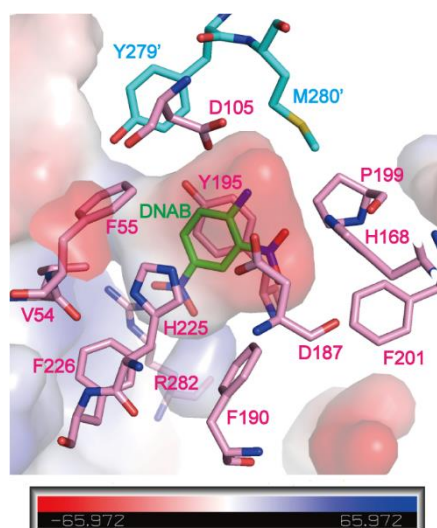


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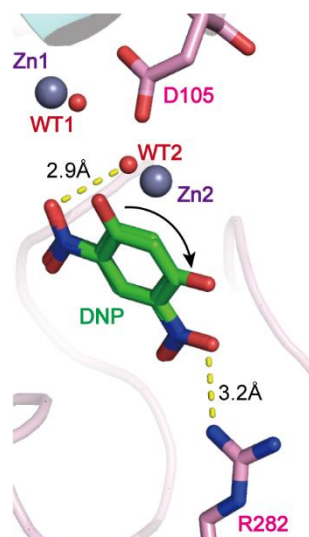
Supplementary Fig. 8 Conversion of DNAN by purified DnhAB in the presence of various transition metal ions. The enzyme (20 μM) was incubated with 0.2 mM of the indicated metal ions, and DNP production was measured using ultraviolet spectrophotometer.

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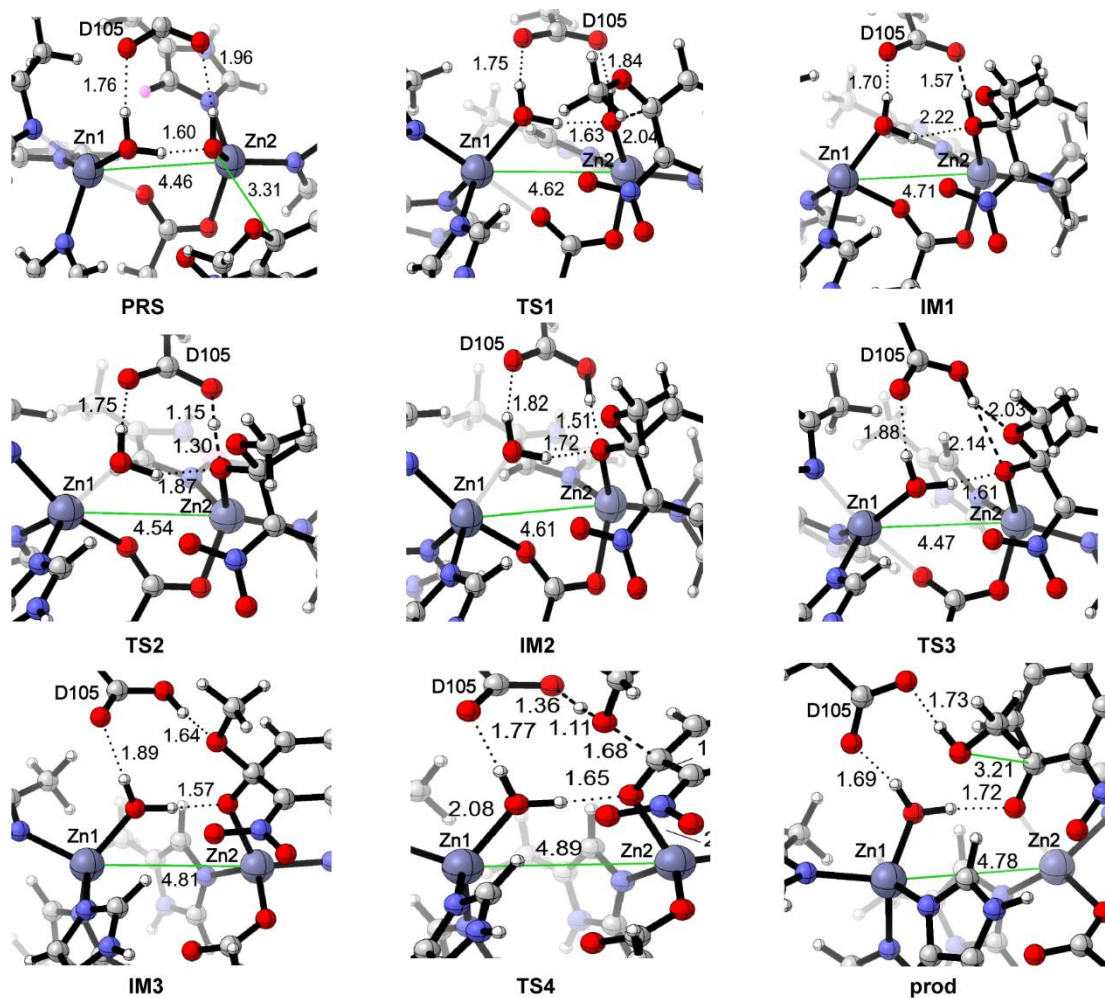
Supplementary Fig. 9 Surface electrostatic potential map of the substrate-binding pocket in DnhAB. The catalytic end of the pocket exhibits prominent negative electrostatic potential, while the opposite end is dominated by a cluster of hydrophobic amino acid residues (V54, F55, F190, F201, and F226).

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Supplementary Fig. 10 Binding modes of DNP in the DnhAB–DNP complex. The two binding modes differ by a 120° rotation of the benzene ring (indicated by the arrow).

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Supplementary Fig. 11 The H_3O_2^- -Asp105 hydrogen-bonding network assists the proton shuttling mechanism. The green straight line marks the distance between the two zinc ions. Black dashed lines show distances between the hydroxide, Asp105, and the substrate ipso-carbon.

Supplementary Table

Supplementary Table 1 Crystallographic data collection and refinement of DnhAB and its complex.

	Apo—DnhAB	DnhAB—DNP	DnhAB—DNAB
PDB ID	43TH	43TG	43TF
Data collection			
Space group	P 1 21 1	P 1 21 1	P 1 21 1
Cell dimensions			
a, b, c (Å)	199.76, 96.42, 121.53	122.47, 113.64, 137.62	121.90, 113.06, 136.87
α , β , γ (°)	90, 103.41, 90	90, 110.34, 90	90, 110.39, 90
Resolution (Å)	94.67-2.31 (2.44-2.31)	29.44-1.98 (2.01-1.98)	80.37-2.13 (2.24-2.13)
R_{merge}	0.12 (0.61)	0.08 (0.87)	0.11 (0.65)
R_{pim}	0.08 (0.48)	0.05 (0.55)	0.07 (0.42)
$I/\sigma I$	9.8 (2.5)	13.4 (2.1)	11.0 (2.3)
Completeness (%)	95.9 (99.5)	99.9 (99.8)	98.6 (99.9)
Redundancy	6.0 (4.9)	6.8 (6.6)	6.7 (6.8)
CC(1/2)	0.99 (0.79)	0.999 (0.831)	0.998 (0.914)
Refinement			
Resolution (Å)	58.25-2.31	29.39-1.98	60.63-2.13
No. reflections	165548	268631	191250
$R_{\text{work}}/R_{\text{free}}$ (%)	18.16/20.54	15.70/17.96	19.89/22.65
No. atoms			
Protein	17105	19542	19029
Ligand/ion	8	76	65
Water	939	2497	1484
B -factors			
Protein	38.32	39.53	45.17
Ligand/ion	33.60	27.91	29.15
Water	37.53	38.13	37.52
R.m.s. deviations			
Bond lengths (Å)	0.003	0.008	0.003
Bond angles (°)	0.690	0.808	0.709
Ramachandran analyses			
Favored (%)	95.90	95.63	95.74
Allowed (%)	4.10	4.21	4.21
Disallowed (%)	0.00	0.16	0.04

Supplementary Table 2 Distances among the active-site metal ion, water molecules, and the substrate ipso carbon in structural data.

	Apo-DnhAB		DnhAB-DNAB		DnhAB-DNP	
	Average	STEDV	Average	STEDV	Average	STEDV
Zn1-Zn2	4.60	0.05	4.64	0.02	4.71	-
H ₂ O1-H ₂ O2	2.87	0.05	2.40	0.15	2.76	-
Zn1-H ₂ O1	2.09	0.06	1.79	0.06	2.00	-
Zn2-H ₂ O2	2.05	0.09	2.20	0.04	2.04	-
Zn1-H ₂ O2	4.54	0.12	3.95	0.09	4.28	-
Zn2-H ₂ O1	3.61	0.15	3.54	0.03	4.01	-
H ₂ O2-C	-	-	2.75	0.08	2.57	-
Zn2-C	-	-	3.93	0.06	3.86	-
H ₂ O1-C	-	-	4.71	0.11	4.88	-
Zn1-C	-	-	5.88	0.01	6.30	-

*C: indicates the ipso-carbon of DNP and DNAB. Since only one chain of DnhAB-DNP exhibits the reactive DNP conformation, distances were calculated solely for this reactive conformation.

Supplementary Table 3 Activity assay of mutants.

Mutant	SA (nMol·min ⁻¹ ·mg ⁻¹)	(%)	Solubility
WT	38434.81±4279.75	93.90±6.34	S
H168A	15.12±2.95	0.04±0.002	S
H103A			NS
H101A			NS
H225A	ND		S
D187A	ND		S
H106A			NS
R282A	ND		S
D105A	ND		S
D105C	ND		S
D105E	ND		S
D105F	ND		S
D105G	ND		S
D105H			NS
D105I	ND		S
D105K	ND		S
D105L	ND		S
D105M	ND		S
D105N	ND		S
D105P	ND		S
D105Q	ND		S
D105R	ND		S
D105S	ND		S
D105T	ND		S
D105V	ND		S
D105W	ND		S
D105Y	ND		S
W34A	33346.43±1516.59	81.47±3.71	S
P36A	381.22±69.5	0.93±0.17	S
D52A			NS
G53A	4480.48±0.92	10.95±2.25	S
V54A			NS
F55A	6544.56±485.47	15.99±1.19	S
W73A			NS
H106A			NS
Y195F	5311.63±15.52	14.01±2.23	S
F226A	1935.41±323.14	4.73±0.79	S
Y279F	30006.86±1337.82	78.78±9.92	S
F190A	8601.71±3120.87	22.82±3.57	S
N196A	217.94±37.75	0.53±0.09	S
V198A	32478.12±1984.77	79.35±4.85	S
F201A	4372.25±650.43	10.68±1.59	S
M280'A	82.90±71.81	0.20±0.18	S

Supplementary Information**Supplementary Table 4 The pH-dependent kinetic analysis.**

pH	7.4	8.0	10.0
$v_{\max}$ ($\mu\text{M}\cdot\text{s}^{-1}$)	3.68 $\pm$ 0.49	5.12 $\pm$ 0.34	9.17 $\pm$ 2.64
k_M	68.96 $\pm$ 10.48	112.53 $\pm$ 8.27	200.94 $\pm$ 70.12
k_{cat} (s^{-1})	10.95 $\pm$ 1.46	15.24 $\pm$ 1.01	27.29 $\pm$ 7.86
k_{cat}/k_M ($\text{nM}\cdot\text{s}^{-1}$)	158.79 $\pm$ 11.94	135.43 $\pm$ 9.28	135.81 $\pm$ 77.98

Supplementary Table 5 Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source
Strains		
<i>Escherichia coli</i> DH5α	<i>supE44lacU169(Φ80lacZΔM15) recA1 endA1 hsdR17 thi-1</i>	Novagen
<i>Nocardioides</i> sp. strain JS1661	DNAN utilizer	This study
<i>Rhodococcus erythropolis</i> strain L88	Lysozyme-sensitive mutant strain of <i>Rhodococcus erythropolis</i> JCM 3201	1
Plasmids		
pTipRT	Shuttle vector <i>E.coli-Rhodococcus</i> ; it has the inducible promoter pTipA and <i>repAB</i> genes for replication in <i>Rhodococcus</i> (from pRE2895); Amp ^r (<i>E. coli</i>) Tet ^R , Thio ^R (<i>Rhodococcus</i>)	2
pTipRT- <i>dnhAB</i>	Derived from pTipRT; it carries the operon <i>dnhAB</i> (within NdeI-HindIII RE sites) under the control of the thiostrepton inducible promoter pTip; Amp ^r (<i>E. coli</i>), Tet ^R , Thio ^R (<i>Rhodococcus</i>)	This study

Supplementary Table 6 Primers used in this study.

Primer	Sequence (5'-3')	Purpose
F	taagaaggagatataccatggGCTGGAGCCATCCACAGTT CGAGAAGGAGAACCTGTACTTCCAGGGTATGA GCGTGACGAGCCAGAC	Amplification of <i>dnhAB</i> gene for expression
R	atgctcgagagatctaagcttTCAGGCTCGGCCGGTGCC	
DnhA-R	atgctcgagagatctaagcttTCACAGCGGCGATCCCAG	Amplification of <i>dnhA</i> gene for expression
DnhB-F	taagaaggagatataccatggGCTGGAGCCATCCACAGTT CGAGAAGGAGAACCTGTACTTCCAGGGTGTGA CCGGCCGGCAGCGC	Amplification of <i>dnhB</i> gene for expression
H168A-F	cccgccgaccgcaggagcg	Amplification of <i>dnhAB</i> mutated at the site of H168
H168A-R	gtccccgtcccTGCggtcgg	
H103A-F	tacaccacgacGCActcgac	Amplification of <i>dnhAB</i> mutated at the site of H103
H103A-R	agtgtggtcgagTGCgtcgtg	
H101A-F	atcatctacaccGCAgaccac	Amplification of <i>dnhAB</i> mutated at the site of H101
H101A-R	gtcgaggtggtcTGCggtgta	
H106A-F	gaccacctcgacGCAactggc	Amplification of <i>dnhAB</i> mutated at the site of H106
H106A-R	tccaaagccagtTGCgtcgag	
H225A-F	tacgtcccggggGCAttctgg	Amplification of <i>dnhAB</i> mutated at the site of H225
H225A-R	aagccgcccagaaTGCccccgg	
D187A-F	ctctacatggccGCAaccgtc	Amplification of <i>dnhAB</i> mutated at the site of D187
D187A-R	ggtgaagacggtTGCggccat	
D105A-F	cacgaccacctcGCAcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105A-R	aaagccagtgtgTGCgaggtg	
D105C-F	cacgaccacctcTGCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105C-R	aaagccagtgtgGCAgaggtg	
D105E-F	cacgaccacctcGAGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105E-R	aaagccagtgtgCTCgaggtg	
D105F-F	cacgaccacctcTTCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105F-R	aaagccagtgtgGAAGaggtg	
D105G-F	cacgaccacctcGGGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105G-R	aaagccagtgtgCCCGaggtg	
D105H-F	cacgaccacctcCACcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105H-R	aaagccagtgtgGTGgaggtg	
D105I-F	cacgaccacctcATCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105I-R	aaagccagtgtgGATgaggtg	
D105K-F	cacgaccacctcAAGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105K-R	aaagccagtgtgCTTgaggtg	

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D105P-F	cacgaccacctcCCGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105P-R	aaagccagtgtgCGGgaggtg	
D105Q-F	cacgaccacctcCAGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105Q-R	aaagccagtgtgCTGgaggtg	
D105R-F	cacgaccacctcCGCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105R-R	aaagccagtgtgGCGgaggtg	
D105L-F	cacgaccacctcCTGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105L-R	aaagccagtgtgCAGgaggtg	
D105M-F	cacgaccacctcATGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105M-R	aaagccagtgtgCATgaggtg	
D105N-F	cacgaccacctcAACcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105N-R	aaagccagtgtgGTTgaggtg	
D105S-F	cacgaccacctcTCGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105S-R	aaagccagtgtgCGAgaggtg	
D105T-F	cacgaccacctcACCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105T-R	aaagccagtgtgGGTgaggtg	
D105V-F	cacgaccacctcGTCcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105V-R	aaagccagtgtgGACgaggtg	
D105W-F	cacgaccacctcTGGcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105W-R	aaagccagtgtgCCAgaggtg	
D105Y-F	cacgaccacctcTACcacact	Amplification of <i>dnhAB</i> mutated at the site of D105
D105Y-R	aaagccagtgtgGTAgaggtg	
F190A-F	gccgacaccgtcGCTaccggg	Amplification of <i>dnhAB</i> mutated at the site of F109
F190A-R	ggtcggcccggtAGCgacggt	
P199A-F	tacaacatcgtgGCTgacttc	Amplification of <i>dnhAB</i> mutated at the site of P199
P199A-R	ccacaggaagtcAGCcacgat	
T194A-F	ttcaccgggccgGCTtacaac	Amplification of <i>dnhAB</i> mutated at the site of P194
T194A-R	cacgatgttgtaAGCcgggccc	
V54A-F	atcaccgacggcgctttccga	Amplification of <i>dnhAB</i> mutated at the site of V54
V54A-R	aagcggttcggaaagcggcgtc	
V198A-F	acctacaacatcGCTcctgac	Amplification of <i>dnhAB</i> mutated at the site of V198
V198A-R	caggaagtcaggAGCgatgtt	
N196A-F	gggccgacctacGCTatcgtg	Amplification of <i>dnhAB</i> mutated at the site of N196
N196A-R	gtcaggcacgatAGCgtaggt	
A186G-F	ttcctctacatgGGGgacacc	Amplification of <i>dnhAB</i> mutated at the site of A186
A186G-R	gaagacgggtgcCCCcatgta	
F201A-F	atcgtgcctgacGCCctgtgg	

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F201A-R	gctcgtccacagGGCgtcagg	Amplification of <i>dnhAB</i> mutated at the site of F201
C276A-F	ttgcgggttgGCCcactac	Amplification of <i>dnhAB</i> mutated at the site of C276
C276A-R	catgtagtagtgGGCcacaac	
F55A-F	accgacggcgtcGCGcgaacg	Amplification of <i>dnhAB</i> mutated at the site of F55
F55A-R	cgcaagcgttcgGCGgacgcc	
H238A-F	ggcgaggtcccgGCGtggtg	Amplification of <i>dnhAB</i> mutated at the site of H238
H238A-R	ctccagcagccaGCGcgggac	
W239A-F	gaggtcccgcacGCCctgctg	Amplification of <i>dnhAB</i> mutated at the site of W239
W239A-R	cggctccagcagGGCgtgcgg	
M280A-F	tgccactactacGCCggtggg	Amplification of <i>dnhAB</i> mutated at the site of M280
M280A-R	ccaccaccaccGGCgtagta	
W34A-F	ccggggtatgagGCCgagccc	Amplification of <i>dnhAB</i> mutated at the site of W34
W34A-R	catggagggtcGGCctcata	
W73F-F	gtcgacaccttcGCCtccccg	Amplification of <i>dnhAB</i> mutated at the site of W73
W73F-R	cgatcccggggaGGCgaaggt	
P36A-F	tatgagtgggagGCCtccatg	Amplification of <i>dnhAB</i> mutated at the site of P36
P36A-R	ggtcgtcatggaGGCctccca	
D52A-F	tggacgatcaccGCCggcgtc	Amplification of <i>dnhAB</i> mutated at the site of D52
D52A-R	tcggaagacgccGGCggtgat	
F226A-F	gtcccggggcatGCCtggcgg	Amplification of <i>dnhAB</i> mutated at the site of F226
F226A-R	cgaagccgccaGGCcatgcc	
G53A-F	acgatcaccgacGCCgtcttc	Amplification of <i>dnhAB</i> mutated at the site of G53
G53A-R	cgttcggaagacGGCgtcggg	

Supplementary Table 7 Calculated energetic parameters of the rate-determining nucleophilic attack step for substrates and non-substrates in the DnhAB catalytic system.

Compounds	Meisenheimer INT	C---O bond constrained optimization	Full optimization (TS1)	Experimental specific activity (mmol·mg ⁻¹ ·min ⁻¹)
Substrates				
DNAN	-7.3 kcal/mol	+9.4 kcal/mol	+9.4 kcal/mol	41.17±5.02
24DNBrB	Unstable and decomposing	--	+5.6 kcal/mol	111.99±14.59
24DNIB	Skipped for iodine basis set	--	--	104.78±5.54
24DNCB	Unstable and decomposing	--	+7.6 kcal/mol	101.07±11.82
24DNFB	-15.1 kcal/mol	--	+4.6 kcal/mol	33.84±6.42
DEPN	-9.8 kcal/mol	+11.3 kcal/mol	+10.6 kcal/mol	6.51±0.65
24DNAB	+7.1 kcal/mol	+10.3 kcal/mol	+12.4 kcal/mol	<0.001
2C4NA	+15.0 kcal/mol	+14.1 kcal/mol	+18.0 kcal/mol	3.60±0.03
12DC4NB	Unstable and decomposing	Fail to converge		1.98±0.02
25DCNB	Unstable and decomposing	+14.8 kcal/mol	+15.6 kcal/mol	0.91±0.09
4F2NBNT	-8.7 kcal/mol	+8.3 kcal/mol	+8.4 kcal/mol	84.85±8.45
4C2NBNT	Unstable and decomposing	+12.3 kcal/mol	+12.4 kcal/mol	6.74±0.61
2C35DNPD	Unstable and decomposing	+6.7 kcal/mol	+6.4 kcal/mol	?
34DNCB	-6.4 kcal/mol	+11.2 kcal/mol	+11.5 kcal/mol	7.32±0.02
26DNCB	Unstable and decomposing	+7.2 kcal/mol	+10.9 kcal/mol	0.32±0.04
Non-substrates				
24DCNB	Unstable and decomposing	High energy, +19.3 kcal/mol		
3N4CTB	Unstable and unformed	High energy, +23.4 kcal/mol		
Anisole	High energy, +37.6 kcal/mol	High energy, +26.9 kcal/mol		
2NA	1.0 kcal/mol	+13.0 kcal/mol	+13.9 kcal/mol	
3NA	High energy, +33.4 kcal/mol	High energy, +22.4 kcal/mol		
4NA	Relative high, 11.9 kcal/mol	High energy, +16.9 kcal/mol	+20.3 kcal/mol	
2CNB	Unstable and decomposing	High energy, +17.7 kcal/mol		
25DNAN	-1.7 kcal/mol	Fail to converge		
124TCB	Unstable and decomposing	Fail to converge		

Supplementary Information

Supplementary reference

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2. Nakashima, N. & Tamura, T. A novel system for expressing recombinant proteins over a wide temperature range from 4 to 35°C. *Biotech & Bioengineering* **86**, 136–148 (2004).