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Synthesis, bioactivity, theoretical and molecular docking study of 1-cyano-*N*-substituted-cyclopropanecarboxamide as ketol-acid reductoisomerase inhibitor

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Abstract—Ketol-acid reductoisomerase (KARI; EC 1.1.1.86) catalyzes the second common step in branched-chain amino acid biosynthesis. The catalyzed process consists of two stages, the first of which is an alkyl migration from one carbon atom to its neighbouring atom. The likely transition state is a cyclopropane derivative, thus a series of new cyclopropane derivatives, such as 1-cyano-*N*-substituted-cyclopropanecarboxamide, were designed and synthesized. Their structures were verified by ^{1}H NMR, FTIR spectrum, MS and elemental analysis. The K_i values of active compounds **2**, **4b** against rice KARI were 95.30 ± 13.71, 207.9 ± 21.99 μM, respectively. The X-ray crystal structure of compound **4a** was also determined. Auto-Dock was used to predict the binding mode of **4a**. This was done by analyzing the interaction of the compounds **4a** with the active sites of spinach KARI. This result was in accord with the result analyzed by the frontier molecular orbital theory.

Plants and most micro-organisms have biosynthetic ability which allows them to survive on relatively simple nutrients. For this reason, plants and microorganisms contain numerous enzymes that are potential targets for designing bioactive compounds such as herbicides and antibiotics. Enzymes involved in the biosynthesis of the branched chain amino acids are one such example. Isoleucine and valine are synthesized in a parallel set of four reactions while an extension of the valine pathway results in leucine.[1]

This pathway is the target for the sulfonylureas,[2] the imidazolinones[3] and a variety of other herbicides,[1] which all inhibit the first enzyme, acetohydroxyacid synthase. The success of these herbicides has stimulated research into inhibitors of other enzymes in the pathway, including the second enzyme in the common pathway,[4] ketol-acid reductoisomerase (KARI; EC 1.1.1.86), and two enzymes in the leucine extension.[5,6] The reaction catalyzed by KARI is shown in Scheme 1 which consists of two steps,[7,8] an alkyl migration followed by a NADPH dependent reduction. Both steps require a divalent metal ion, such as Mg^{2+}, Mn^{2+} or Co^{2+}, but the alkyl migration is highly specific for Mg^{2+}. HOE 704[9] and IpOHA[10] are potent competitive inhibitors of the enzyme (Scheme 1).

A transition state being a cyclopropane is postulated and mimicked by Gerwick et al.[11] They showed that cyclopropane-1,1-dicarboxylate (CPD) can inhibit *Escherichia coli* KARI. They also showed that application of CPD to various plant tissues caused the accumulation of the substrate 2-acetolactate; in vivo data strongly suggest that the CPD can inhibit the activity of KARI Scheme 1.[12]

The first step in the KARI catalyzed process involves an alkyl migration from one carbon atom to its neighboring atom. The likely transition state is a cyclopropane derivative. For this reason, some new cyclopropane derivatives were synthesized in our laboratory (Scheme 2).

Biological studies revealed that some of these compounds inhibit ketol-acid reductoisomerase in vivo effectively.

Keywords: Cyclopropanecarboxamide; Synthesis; Structure; Molecular docking; Theoretical study; KARI.
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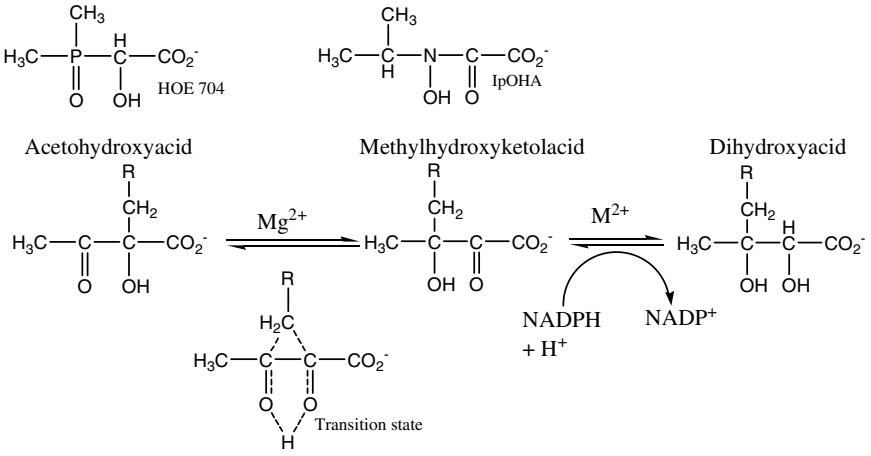
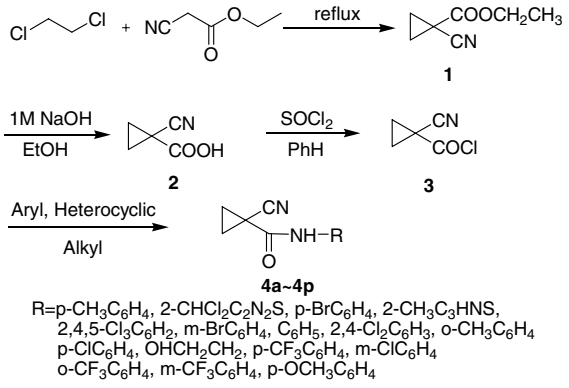


Scheme 1. Reaction catalyzed by KARI.



Scheme 2. Synthesis route for compounds **4a**–**p**.

The 1-cyan-1-cyclopropane carboxylic acid, prepared from 1,2-dichloroethane and ethyl cyanacetate was cyclized for 16 h at refluxing temperature. In order to optimize the reaction time, microwave assisted irradiation was applied which shortened the reaction time to 40 min. Compound **3** reacted with substituted anilines, heterocyclic amine or alkyl amines in the presence of inorganic base to yield substituted cyclopropanecarboxamides as shown in Scheme 2.[13]

The KARI activities in vitro of these compounds were determined.[14] The results for compound, 1, 2 and compounds **4a**–**p** are summarized in Table 1.

It was found from Table 1 that compounds **2**, **4b**, **4h**, **4i**, **4j** and **4k** have favourable inhibitory activity against KARI. The data given in Table 1 indicated that the change of substituent at phenyl ring affects the KARI activity. When the benzene ring is substituted by CF_3 group, the compounds generally have no KARI bioactivity, as **4l**, **4n**, **4o**. While for heterocyclic and alkane substituents, their inhibitory activities increase for **4b** and **4k**. For the compounds, **2**, **4a** and **4b**, further bioassay was conducted and their K_i values against KARI were 95.30 ± 13.71, >300 and 207.9 ± 21.99 μM, respectively. Hence, these identified cyclopropane derivatives could be useful for further optimization work in finding the potential KARI inhibitors.

Table 1. Inhibition rate (%) of compounds **4a**–**p** against rice KARI at 200 ppm in vitro

Compound	R	KARI activity
1		0
2		100
4a	*p*-$CH_3C_6H_4$–	61.21
4b	2-$CHCl_2C_2N_2S$–	100
4c	*p*-BrC_6H_4–	32.23
4d	2-CH_3C_3HNS–	69.81
4e	2,4,5-$Cl_3C_6H_2$–	0
4f	*m*-BrC_6H_4–	17.25
4g	C_6H_5–	77.23
4h	2,4-$Cl_2C_6H_3$–	97.04
4i	*o*-$CH_3C_6H_4$–	100
4j	*p*-ClC_6H_4–	93.92
4k	$OHCH_2CH_2$–	98.92
4l	*p*-$CF_3C_6H_4$–	0
4m	*m*-ClC_6H_4–	0
4n	*o*-$CF_3C_6H_4$–	0
4o	*m*-$CF_3C_6H_4$–	0
4p	*p*-$OCH_3C_6H_4$–	3.95

In order to study the structure–activity relationship, the single-crystal structure of **4a** was determined[15] by X-ray crystallography[16] as illustrated in Figure 1 in which three C–N bond lengths C(5)–N(2), C(6)–N(2) and N(1)–C(1) are 0.135, 0.142, and 0.114 nm, respectively, which are all longer than that of 0.134 nm in the single heterocycle ring.[17] In **4a**, the bond length of C(1)–N(1) is 0.1396 nm, which is longer than the double C–N bond.[18] Based on the computal results by Gaussian,[19,20] it was seen that DFT, HF and MP2 have good coherence with the crystal diffraction, for example it can be observed that C(2)–C(5) > C(1)–C(2) > N(2)–C(6) > N(2)–C(5) >O(1)–C(5) > N(1)–C(1) in crystal structure, which is accordance with the order of C(2)–C(5) >C(1)–C(2) > N(2)–C(6) > N(2)–C(5) > O(1)–C(5) > N(1)–C(1) in all calculation structures.

According to the frontier molecular orbital theory, HOMO and LUMO are two most important factors which affect the bioactivities of compounds. HOMO has the priority to provide electrons, while LUMO accepts electrons first.[21,22] Thus, study on the frontier

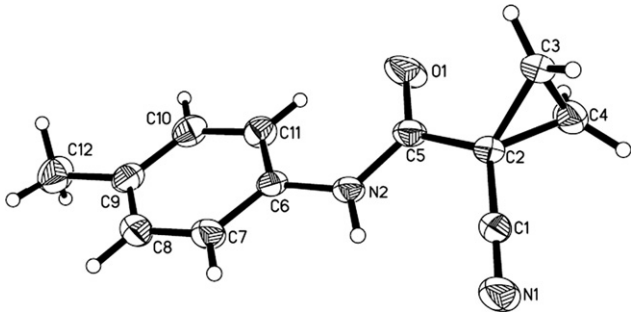


Figure 1. Molecular structure of **4a**.

orbital energy can provide some useful information for the active mechanism. Taking HF (Hatree–Fork) results, the geometry of the frame of **4a** is hardly influenced by the introduction of either the cyano group or the cyclopropane ring from Figure 2. The HOMO of **4a** is mainly located on aromatic ring and the amide group. On the other hand, the LUMO of **4a** contains aromatic ring, the amido group, the cyano group and the cyclopropane ring. The fact that **4a** has strong affinity suggests the importance of the frontier molecular orbital in the π–π stacking or hydrophobic interactions. This also implies that the orbital interaction between **4a** and the rice KARI amino acid residues is dominated by π–π or hydrophobic interaction between the frontier molecular orbitals.

To make the results predicted by our frontier molecular orbital model more relevant to the active sites of the enzyme and to further explore a probable binding site in the KARI, the compound **4a** was docked[21,22] into the active sites of KARI.[23]

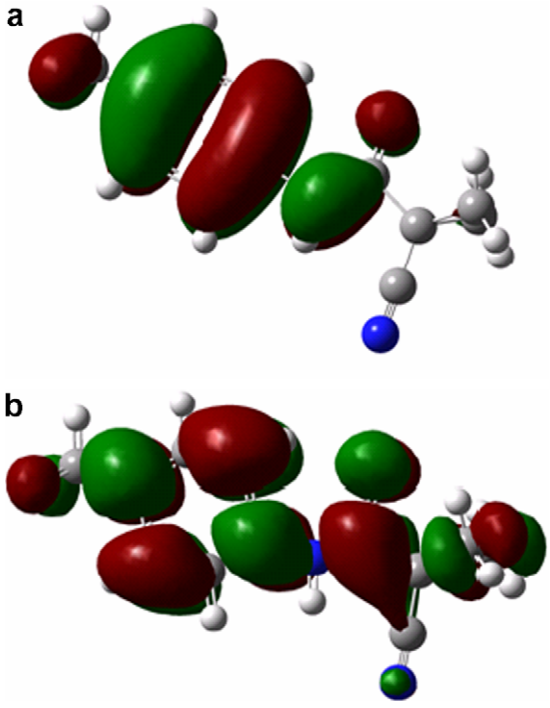


Figure 2. Frontier molecular orbitals of compound **4a**: (a) HOMO of compound **4a**; (b) LUMO of compound **4a**.

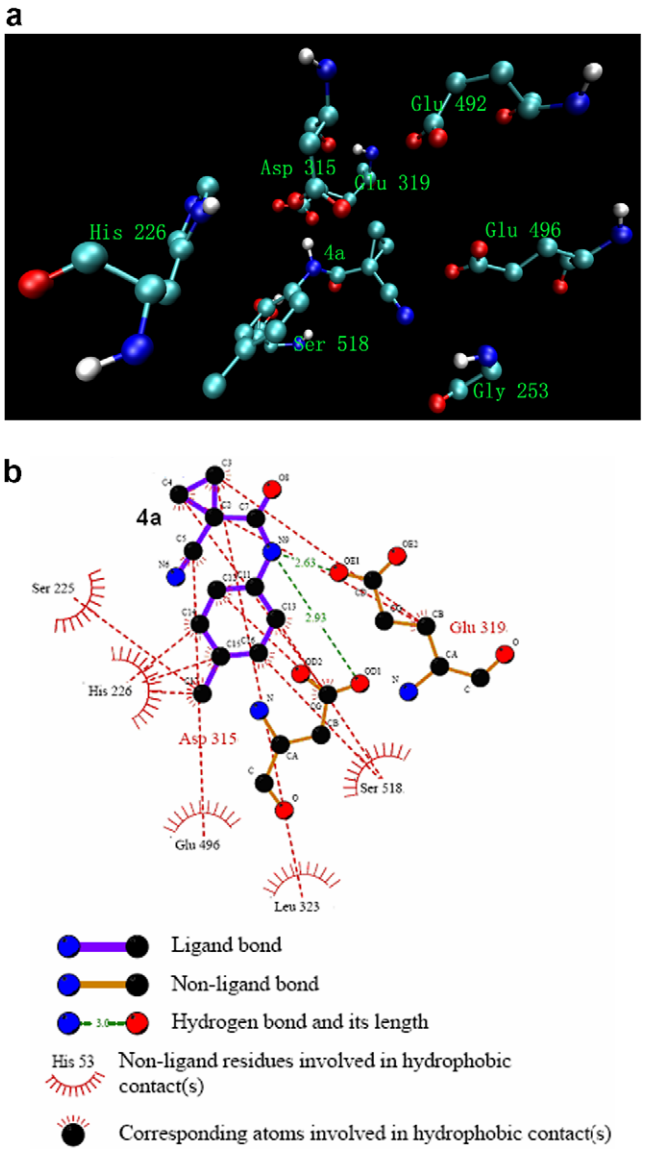


Figure 3. Binding modes of compound **4a** in the active sites of spinach KARI: (a) π–π stacking interaction between the His 226 side chain and phenyl ring; (b) hydrogen bond and hydrophobic interaction between 4a and the rice KARI amino acid residues.

Visual inspection of the conformation of **4a** docked into the KARI binding site revealed that the phenyl rings are hosted in the pocket of KARI and are oriented to establish π–π stacking interactions with the His 226 side chains (Fig. 3a). Moreover, two hydrogen bonds between the amino groups of **4a** and the carbonyl oxygen of Glu 319 and Asp 315 side chain are also observed. Furthermore, the cyclopropane ring and aromatic ring are embedded in a large hydrophobic pocket formed by Ser 225, His 226, Glu 496, Leu 323, Ser 518, Glu 319 and Asp 315 (Fig. 3b).

Acknowledgments

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13. General procedure: ethyl cyanacetate (22.6 g, 0.2 mol), 1,2-dichloroethane (160 g, 0.2 mol), potassium carbonate (220 g, 1.6 mol) and catalytic amount of Bu4NHSO4 (1.0 g) were vigorously refluxed in 1,2-dichloroethane for 6 h after which the reaction mixture was poured into water (800 mL). The product was extracted with ether (5× 100 mL), combined extracts were dried over $MgSO_4$ and then the solvent was removed on a rotary evaporator and the residue was distilled under pressure: bp 115–118/15 mmHg. Yield 85%. ^{1}H NMR(δ, $CDCl_3$):1.30–1.34 (t, J = 7.14 Hz, 3H, CH3), 1.59–1.69 (m, J = 3.27 Hz, 4H, cyclopropane–CH_2), 4.21–4.27 (q, J = 7.13 Hz, 2H, CH_2). An ester (0.03 mol) was added to a ca. 15% aqueous solution containing 3 mol equivalents of sodium hydroxide and the suspension was vigorously stirred at ambient temperature for 2 days until a homogeneous solution was formed. The solution was extracted with ether (2× 50 mL) to remove traces of unreacted ester, the water phase was acidified with concentrated hydrochloric acid and a free acid was extracted with ether (3× 100 mL). The combined extracts were dried over $MgSO_4$ and then the solvent was removed on a rotary evaporator. Yields 51%. Mp 88–90 °C.

    To a benzene solution (25 mL) of cyanocyclopropanecarboxylic acid (7.50 mmol) was added thionyl chloride (30 mmol) and the mixture was refluxed for 2 h to give acid chloride.

    Preparation of compound **4a**. Dropwised the acid chloride to substituted *p*-toluidine (7.50 mmol), then vigorously stirred at ambient temperature for 4 h. The yield was 84% with mp (106–108) °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.58–1.79 (m, 4H, CH_2), 2.92 (s, 3H, CH_3), 7.15–7.39 (m, 4H, ArH), 7.96 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 14.35 (s, 2C, CH_2), 18.44 (s, 1C, C), 21.13 (s, 1C, CH_3), 120.30 (s, 1C, CN), 120.81 (s, 2C, ArC), 129.82 (s, 2C, ArC), 163.48 (s, 1C, CO), 135.40 (d, 1C, ArC); IR (KBr) v: 3338 (–NH), 3107, 3035, 2920 (cyclopropane, CH), 2243 (C ≡ N), 1898 (C═O), 1689,1602, 1523 (Ar C–C), 810 cm^{-1}, ESI-MS (41 eV): m/z: 199.6, 65.96.

    Compound **4b**. A white solid, yield 82%; mp 109–117 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.73–1.80 (m, 4H, CH_2), 6.26 (s, 1H, hetero-H), 7.02 (s, 1H, NH); IR (cm^{-1}) 3336, 3184, 3077, 2991, 2268, 1731. ESI-MS: 273.48, 230.08, 204.43, 141.20.

    Compound **4c**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.59–1.72 (m, 4H, CH_2), 7.15–7.39 (m,J = 8.784 Hz, 4H, ArH), 8.05 (s, 1H, NH); IR (cm^{-1}) 3347, 3093, 2236, 1692, 1598, 1525, 1489, 814. ESI-MS: 264.98, 197.95, 116.02, 80.99. Elemental analysis: C, 49.80; H, 3.28; N, 10.58; calculated from $C_{12}H_9BrN_2O$. Observed: C, 49.84; H, 3.42; N, 10.57.

    Compound **4d**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M)1.26(s,3H,CH_3), 1.73–1.94(m, 4H, CH_2), 7.81(d,1H, Heterocycle); IR (cm^{-1}) 3450, 3077, 2991, 2855, 2268, 1731.ESI-MS: 206.37.

    Compound **4e**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.62–1.84 (m, 4H, CH_2), 7.52 (s, 1H, ArH), 8.50 (s, 1H, ArH), 8.68(s, 1H, NH); IR (cm^{-1}) 3369, 3122, 3020, 2229, 1692, 1576, 1496, 1456, 879. ESI-MS: 287.22, 219.95, 253.84, 222.10, 183.72. Elemental analysis: C, 45.82; H, 2.21; N, 9.41; calculated from $C_{11}H_7Cl_3N_2O$. Observed: C, 45.63; H, 2.44; N, 9.67.

    Compound **4f**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.61–1.83 (m, 4H, CH_2), 7.15 (d, J = 7.573 Hz, 1H, ArH), 7.31 (d, J = 3.893 Hz, 1H, ArH), 7.66 (d, J = 1.938 Hz, 1H, ArH), 8.50 (s, 1H, ArH), 8.68(s, 1H, NH); IR (cm^{-1}) 3320, 3195,3115, 2244, 1680, 1590,1527, 1476, 883,810,766,656.

    Compound **4g**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.59–1.81 (m, 4H, CH_2), 7.18 (t, J = 6.848 Hz, 1H, ArH), 7.36 (t, J = 7.933 Hz, 2H, ArH), 7.50 (d, J = 8.293 Hz, 2H, ArH), 8.03 (s, 1H, NH); IR (cm^{-1}) 3249, 3191,3127, 2239, 1680, 1595,1536, 1488, 751,693. ESI-MS: 185.41, 65.97. Elemental analysis: C, 70.94; H, 5.41; N, 15.39; calculated from $C_{11}H_{10}N_2O$. Observed: C, 70.95; H, 5.41; N, 15.04.

    Compound **4h**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.63–1.82 (m, 4H, CH_2), 7.28 (d, J = 2.270 Hz, 1H, ArH), 7.44 (d, J = 2.297 Hz, 1H, ArH), 7.28 (d, J = 8.888 Hz, 1H, ArH), 8.67(s, 1H, NH); IR (cm^{-1}) 3398, 3115, 2236, 1699, 1583, 1510, 959, 923, 821, 727. ESI-MS: 253.29, 185.98, 149.81, 114.05. Elemental analysis: C, 51.70; H, 3.21; N, 10.79; calculated from $C_{11}H_8Cl_2N_2O$. Observed: C, 51.79; H, 3.16; N, 10.98.

    Compound **4i**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.59–1.81 (m, 4H, CH_2), 7.08 (t, J = 6.815 Hz, 2H, ArH), 7.18 (d,J = 5.187 Hz, 1H, ArH), 7.77 (d, J = 8.998 Hz, 1H, ArH), 7.99 (s, 1H, NH); IR (cm^{-1}) 3435, 3105,3019, 2225, 1695, 1588, 1531,1459, 758. ESI-MS: 200.15, 171.18, 106.16, 77.11.

    Compound **4j**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.62–1.81 (m, 4H, CH_2), 7.32 (d, J = 8.794 Hz, 2H, ArH), 7.47 (d, J = 8.856 Hz, 2H, ArH), 8.03 (s, 1H, NH); IR (cm^{-1}) 3331, 3120,2941, 2247, 1667, 1593, 1534,1490, 832.ESI-MS: 219.42, 152.06, 116.04. Elemental analysis: C, 59.66; H, 3.96; N, 12.58; calculated from $C_{11}H_9ClN_2O$. Observed: C, 59.88; H, 4.11; N, 12.70.

    Compound **4k**. A white solid, yield 93%; m.p. 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.24 (t, 1H, OH), 1.51–1.71(m, 4H, CH_2), 3.62 (t, 2H, J = 5.380 Hz, NH–CH_2), 4.17 (q, 2H, J = 5.239 Hz, OH–CH_2), 6.88 (s, 1H, NH); IR (cm^{-1}) 3356, 3195,3115, 2251, 1698, 1588, 1534,1486, 832.

    Compound **4l**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.64–1.84 (m, 4H, CH_2), 7.60 (d, J = 9.086 Hz, 2H, ArH), 7.65 (d, J = 9.071 Hz, 2H, ArH), 8.17 (s, 1H, NH); IR (cm^{-1}) 3500,3327, 3120, 2239, 1674, 1617, 1567,1495, 808. ESI-MS: 287.34(M+Na), 160.33.

Compound **4m**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.63–1.83 (m, 4H, CH_2), 7.47 (dd, J = 7.530 Hz, 2H, ArH), 7.65 (d, J = 7.631 Hz, 1H, ArH), 7.89 (s, 1H, ArH), 8.12 (s, 1H, NH); IR (cm^{-1}) 3313, 3198,3120, 2261, 1681, 1602, 1538,1474, 872, 765,672. ESI-MS: 219.33, 152.02.

Compound **4n**. A white solid, yield 93%; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300 M) 1.61–1.82 (m, 4H, CH_2), 7.55 (d, J = 7.361 Hz, 1H, ArH), 7.65 (dd, J = 7.793 Hz, 1H, ArH), 7.94 (d, J = 8.150 Hz, 1H, ArH), 8.48 (d, J = 8.065 Hz, 1H, ArH), 6.64 (s, 1H, NH); IR (cm^{-1}) 3312, 3188,3115, 2244, 1680, 1593, 1527,1476, 766. ESI-MS:285.63(M+Na), 194.94, 117.05.

Compound **4o**. A white solid, yield 93%; mp 106–108 °C; 1HNMR ($CDCl_3$, 300 M) 1.61–1.81 (m, 4H, CH_2), 7.15 (m, J = 7.626 Hz, 1H, ArH), 7.30(dd, J = 7.894 Hz, 1H, ArH), 7.51 (d, J = 8.841 Hz, 1H, ArH), 7.66 (d, J = 1.914 Hz, 1H, ArH), 8.05 (s, 1H, NH); IR (cm^{-1}) 3334, 3127,3062, 2247, 1688, 1602, 1531,1476, 844.ESI-MS:253.22,186.20, 166.02, 145.82. Elemental analysis: C, 59.41; H, 4.12; N, 12.13; calculated from $C_{11}H_9ClN_2O$. Observed: C, 59.88; H, 4.11; N, 12.70.

Compound **4p**. A white solid, 93% yield; mp 106–108 °C; ^{1}HNMR ($CDCl_3$, 300M) 1.57–1.81 (m, 4H, CH_2), 3.80 (s, 3H, CH3), 6.88 (d, J = 9.015 Hz, 2H, ArH), 7.04(d, J = 9.001 Hz, 2H, ArH), 7.94 (s, 1H, NH); IR (cm^{-1}) 3334, 3195,3115, 2251, 1680, 1600, 1534,1490, Elemental analysis: C, 66.59; H, 5.51; N, 12.96; calculated from $C_{12}H_{12}N_2O_2$. Observed: C, 66.65; H, 5.59; N, 12.96.

14. KARI activity was measured by following the decrease in A340 at 30 °C in solutions containing 0.2 mM NADPH, 1 mM $MgCl_2$, substrate (2-acetolactate or hydroxypyruvate) and CPD or other inhibitors as required, in 0.1 M Tris–HCl, pH 8.0. The reaction was started by adding the enzyme except for inhibitor preincubation experiments, where the substrate was added last.
15. Crystal data of **4a**. $C_{12}H_{12}N_2O$, M = 200.24, Monoclinic, a = 7.109(4), b = 13.758(7), c = 11.505(6) Å, β = 102.731(8)°, V = 1097.6(9) Å^3, T = 294(2) K, space group P2(1)/c, Z = 4, Dc = 1.212 g/cm^3, μ (Mo–Kα) = 0.71073 mm^{-1}, F (0 0 0) = 424. 6109 reflections measured, 2290 unique (R_{int} = 0.0294), which were used in all calculation. Fine R_1 = 0.0490, wR (F^2) = 0.1218 (all data). Full crystallographic details of **4a** have been deposited at the Cambridge Crystallographic Data Center and allocated the deposition number CCDC 612888.
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19. According to the above crystal structure, a crystal unit was selected as the initial structure, while HF/6-31G (d,p), DFT-B3LYP/6-31G(d,p) and MP2/6-31G(d,p) methods in Gaussian 03 package were used to optimize the structure of the title compound. Vibration analysis showed that the optimized structures were in accordance with the minimum points on the potential energy surfaces, which means no virtual frequencies, proving that the obtained optimized structures were stable. All the convergent precisions were the system default values, and all the calculations were carried out on the Nankai Stars supercomputer at Nankai University.
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22. All docking procedures were done in NanKai Stars supercomputer at Nankai University. The automated molecular docking calculations were carried out using AutoDock 3.05. The AUTOTORS module of AutoDock defined the active torsions for each docked compound. The active sites of the protein were defined using AutoGrid centred on the IpOHA in the crystal structure. The grid map with $60 \times 60 \times 60$ points centred at the center of mass of the KARI and a grid spacing of 0.375 Å was calculated using the AutoGrid program to evaluate the binding energies between the inhibitors and the protein. The Lamarckian genetic algorithm (LGA) was used as a searching method. Each LGA job consisted of 50 runs, and the number of generation in each run was 27000 with an initial population of 100 individuals. The step size was set to 0.2 Å for translation and 5° for orientation and torsion. The maximum number of energy evaluations was set to 15,00,000. Operator weights for cross-over, mutation and elitism were 0.80, 0.02 and 1, respectively. The docked complexes of the inhibitor-enzyme were selected according to the criterion of interaction energy combined with geometrical and electronic matching quality.
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