Bioorganic & Medicinal Chemistry Letters 19 (2009) 5586–5589

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Kojic acid–amino acid conjugates as tyrosinase inhibitors

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ARTICLE INFO

Article history:
Received 13 May 2009
Revised 10 August 2009
Accepted 11 August 2009
Available online 14 August 2009

Keywords:
Kojic acid
Tyrosinase inhibitor
Stability
Enzyme kinetics
AUTODOCK program

ABSTRACT

Kojic acid (KA), a well known tyrosinase inhibitor, has insufficient inhibitory activity and stability. We modified KA with amino acids and screened their tyrosinase inhibitory activity. Among them, kojic acid–phenylalanine amide (KA-F-NH_2) showed the strongest inhibitory activity, which was maintained for over 3 months at 50 °C, and acted as a noncompetitive inhibitor as determined by kinetic analysis. It also exhibited dopachrome reducing activity. We also propose a new tyrosinase inhibition mechanism based on the docking simulation data.

Melanin is a dark pigment produced by skin cells in the innermost layer of the epidermis. Melanogenesis is initiated with the first step of tyrosine oxidation by tyrosinase. When the skin is exposed to UV radiation, the formation of abnormal melanin pigment occurs, which constitutes a serious esthetic problem that is particularly prevalent in middle-aged and elderly individuals.[1,2] Tyrosinase plays an important role in the pathway of melanin biosynthesis from tyrosine. It catalyzes two distinct reactions involving molecular oxygen; the hydroxylation of tyrosine to L-DOPA as monophenolase and the oxidation of L-DOPA to dopaquinone as diphenolase. Dopaquinone is nonenzymatically converted to dopachrome, and ultimately to dihydroxyindols which cause the production of melanin pigments.[3] Therefore, the development of tyrosinase inhibitors is of great concern in the medical, agricultural, and cosmetic fields.[4] Among the many kinds of tyrosinase inhibitors, kojic acid (KA) has been intensively studied. It acts as a good chelator of transition metal ions such as Cu^{2+} and Fe^{3+} and a scavenger of free radicals.[5] This fungal metabolite is currently applied as a cosmetic skin-lightening agent and food additive to prevent enzymatic browning.[6] KA shows a competitive inhibitory effect on the monophenolase activity and a mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase.[7,8] However, its use in cosmetics has been limited, because of the skin irritation caused by its cytotoxicity and its instability during storage. Accordingly, many KA derivatives have been synthesized to improve its properties by converting the C-7 hydroxyl group into an ester,[10] hydroxyphenyl ether,[11] glycoside,[12] amino acid derivatives, or tripeptide derivatives.[13,14,16]

Recently, our group introduced various KA-tripeptide derivatives as new tyrosinase inhibitors. In this study, we found that KA-tripeptide amides, especially KA-FWY-NH_2, showed similar tyrosinase inhibitory activities to those of KA-tripeptide free acids but exhibited superior storage stability than those of KA and KA-tripeptide free acids.[15,16] During these studies, we noticed the importance of C-terminal amide form in KA-peptide conjugates. To find further KA derivatives with higher tyrosinase inhibitory activity, stability, and synthetic efficiency, we prepared a library of KA-amino acid amides (KA-AA-NH_2) and screened their tyrosinase inhibitory activities.

KA was activated with 1,1′-carbonyldiimidazole (CDI) and coupled to the resin-bounded amino acids. After cleavage of the KA-AA-NH_2 from the resin, it was analyzed by HPLC and characterized by MALDI-TOF mass spectroscopy.[17–19] Twenty kinds of KA-AA-NH_2 were examined for their tyrosinase inhibitory activity on mushroom tyrosinase (Fig. 1).

As expected, most of them showed better tyrosinase inhibitory activity than KA itself. Especially, when amino acids which possess aromatic side chains, such as phenylalanine, tryptophan, tyrosine, and histidine, were conjugated to KA, its tyrosinase inhibitory activity was dramatically increased to over 90% at 20 μM. Especially, the inhibitory activity of KA-F-NH_2 toward mushroom tyrosinase was 98.6%, its IC_{50} value in the inhibition of L-DOPA oxidation was 14.7 μM and K_i value was 11.0 μM. In case of KA, its tyrosinase inhib-

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doi:10.1016/j.bmcl.2009.08.041

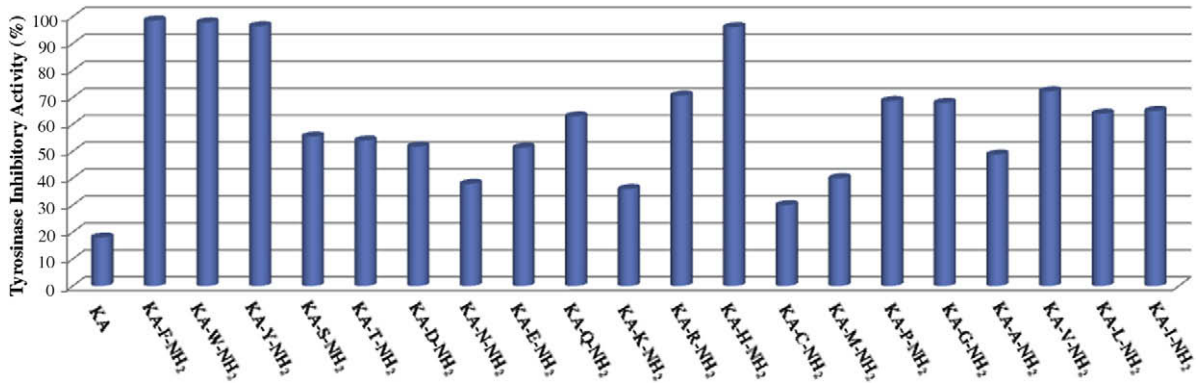

Figure 1. Tyrosinase inhibitory activities of KA and KA-AA-NH_2. The reaction media contained L-tyrosine (1.67 mM), mushroom tyrosinase (2 units/μl) and tyrosinase inhibitor (20 μM) in a total volume of 750 μl of 0.1 M phosphate buffer (pH 6.8). The data were obtained by measuring the UV absorbance at 475 nm.

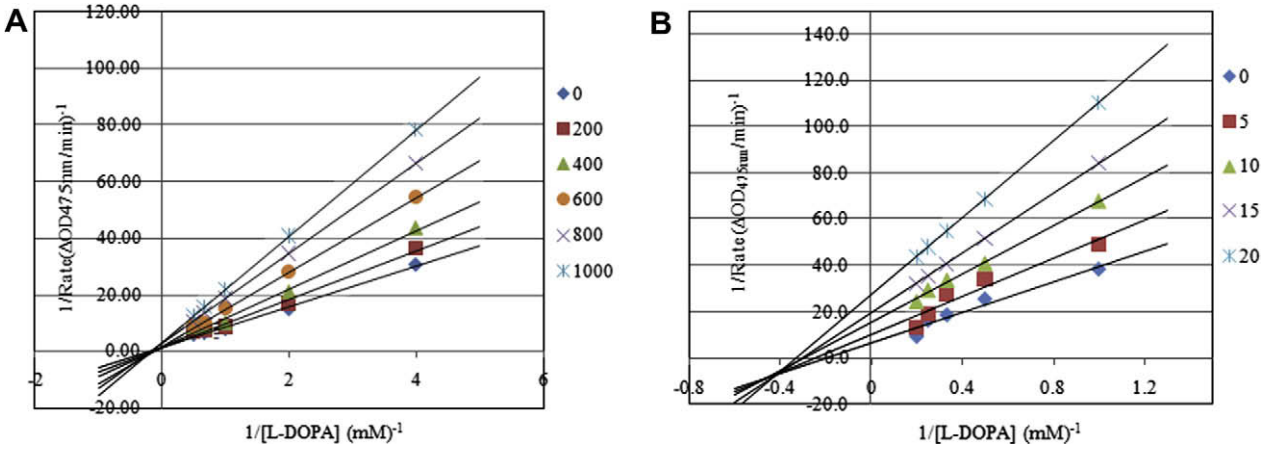

Figure 2. Lineweaver–Burk plots of mushroom tyrosinase with L-DOPA as a substrate in the presence of KA (A), and KA-F-NH_2 (B). The data were obtained from triplicate runs.

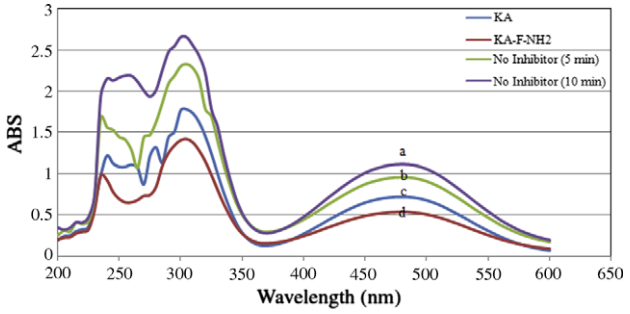

Figure 3. Absorption spectra of reaction solution of: (a) mushroom tyrosinase and L-tyrosine after 10 min of reaction; (b) after 5 min of reaction; (c) after adding KA to b; (d) after adding KA-F-NH_2 to b.

itory activity was 18.3%, IC_{50} value was 722.0 μM and K_i value was 582.7 μM. Kinetic study of the inhibition of L-DOPA oxidation by KA and KA-F-NH_2 revealed that the V_{max} values of mushroom tyrosinase activity was increased as the concentration of inhibitors was increased, while the K_m values remained unchanged (Fig. 2).[20–24] Therefore, KA-F-NH_2 is regarded as a non-competitive inhibitor of mushroom tyrosinase as KA.

Tyrosinase is an enzyme that catalyzes the hydroxylation of L-tyrosine and the subsequent oxidation of L-DOPA to dopaquinone. When L-tyrosine aqueous solution was incubated with tyrosinase at 37 °C, its color turned to red-brown due to dopachrome formation. When KA was added during the reaction, the color intensity of the solution became fainter than before adding. To evaluate its activity, the reaction mixture was scanned by a spectrophotometer (Fig. 3). Two distinct absorption peaks below 350 nm were generated by the reaction of L-tyrosine and L-DOPA with tyrosinase. The reaction mixture revealed an absorption peak at 475 nm which corresponded to the formation of dopachrome.[8] When the reaction solution of tyrosinase and L-tyrosine was incubated for 10 min, the intensity of the absorption peak at 475 nm was increased to a greater extent than that of the reaction solution

Table 1
Effect of KA and KA-AA-NH_2 on dopachrome concentration

	ABS (475 nm)	Dopachrome production reducing activity[d] (%)
No inhibitor (5 min)	0.995[a]	
No inhibitor (10 min)	1.053[b]	
KA	0.973[c]	8
KA-F-NH_2	0.730[c]	31
KA-W-NH_2	0.797[c]	24
KA-Y-NH_2	0.910[c]	14
KA-H-NH_2	0.880[c]	16

[a] Measured after the reaction mixture of tyrosinase and L-tyrosine reacted for 5 min.
[b] Measured after the reaction mixture of tyrosinase and L-tyrosine reacted for 10 min.
[c] Measured after 5 min of inhibition addition into the reaction mixture of tyrosinase L-tyrosine reacted for 5 min.
[d] Calculated using the following formula: $[(A - B)/A] \times 100$ (A, absorbance of No inhibitor (10 min); B, absorbance of test inhibitor solution).

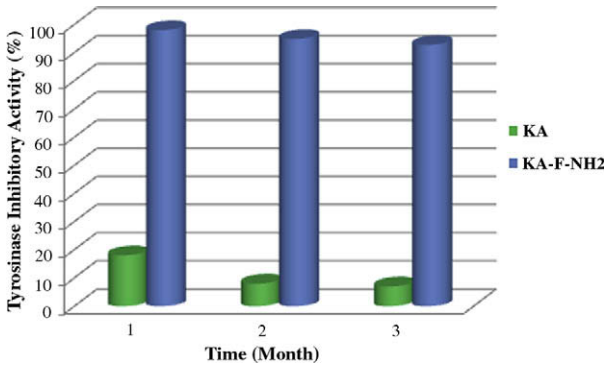

Figure 4. Storage stabilities of KA and KA-F-NH_2. (20 μM dissolved in DI water) were stored at 50 °C for 3 months and their tyrosinase inhibitory activity was measured by Mushroom Tyrosinase Inhibition Method).

reacted for 5 min. This means that intermediate products in the melanin biosynthetic pathway were continuously synthesized as time went by. However, when KA or KA-F-NH_2 was added as a tyrosinase inhibitor, the absorption peak at 475 nm, corresponding to dopachrome formation, was decreased (Table 1). It has been reported that the KA can act as a reducing agent of dopaquinones to L-DOPA.[8,9] Although it is unclear that KA or KA-F-NH_2 have reduced the dopachrome or conjugated with dopachrome, forming a complex, we measured the dopachrome reducing activity of KA-AA-NH_2 in the same way as we employed in the Mushroom Tyrosinase Inhibition Assay. From this, we confirmed that KA-F-NH_2 was more effective in reducing the melanin biosynthetic pathway products than KA itself or other KA-AA-NH_2 derivatives. When the reducing activity of the amount of dopachrome production was calculated in the same way as that for tyrosinase inhibitory activity, the dopachrome production reducing activity of KA was only 8%. However, the reducing activities of KA-F-NH_2, KA-W-NH_2, KA-Y-NH_2, and KA-H-NH_2 were calculated to be 31%, 24%, 14%, and 16%, respectively. The dopachrome production reducing activity of KA-F-NH_2 was about fourfold higher than that of KA. From this observation, we concluded that KA-AA-NH_2 decreased the amount of dopachrome much better than KA and delayed melanin formation.

When the C-terminus of the peptide was converted to the amide form, its stability as a function of time, temperature, and

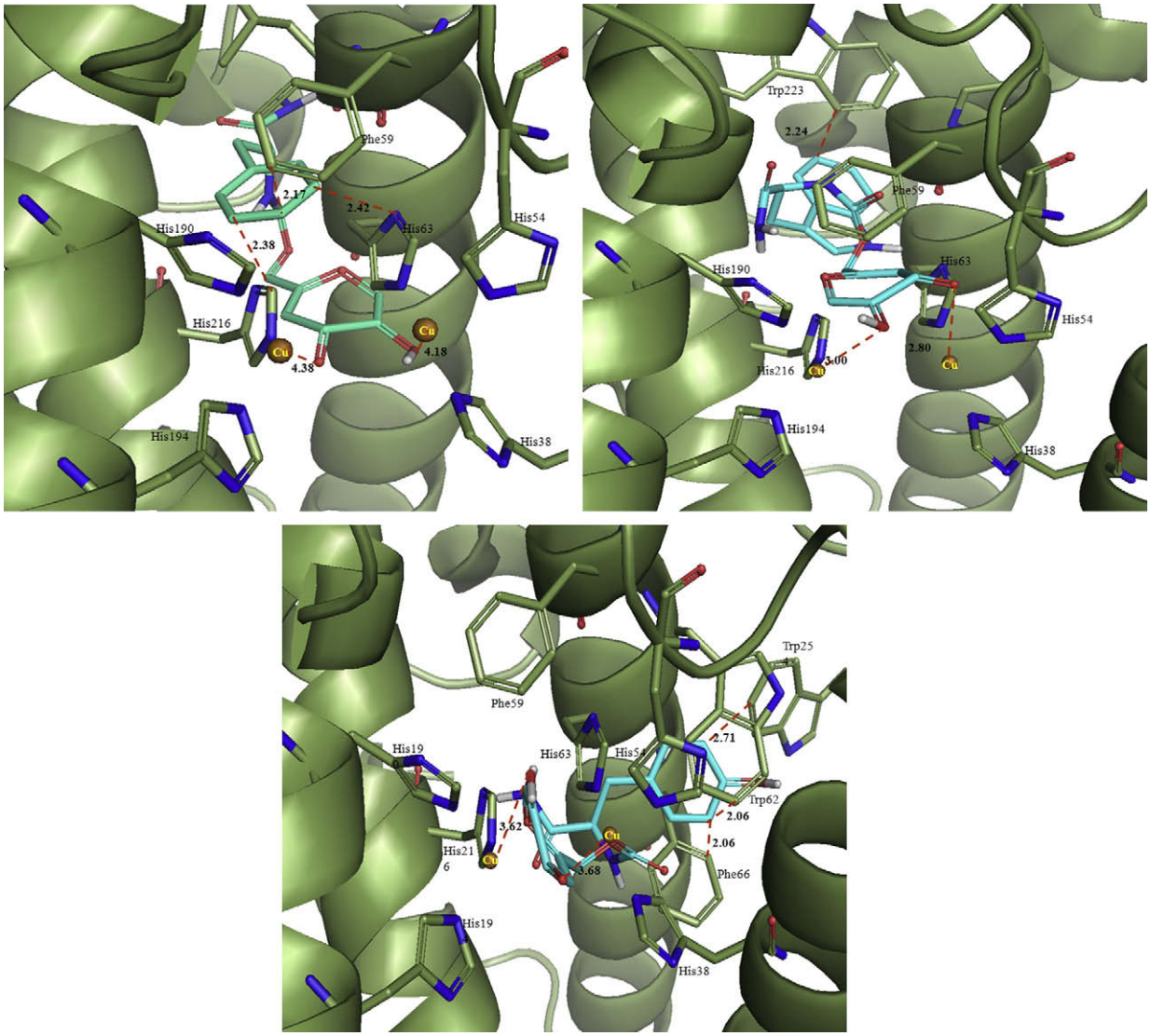

Figure 5. Molecular interactions of KA-AA-NH_2 near the tyrosinase binding site after docking simulations (Matoba, Y. et al. *J. Biol. Chem.* **2006**, *281*, 8981). (Left up) The interaction of the phenylalanine group of KA-F-NH_2 with His63, His216 and Phe59 near the enzyme binding site is shown with their distances; red = oxygen, blue = nitrogen (Right up) KA-W-NH_2 interaction in tyrosinase active site. (Down) KA-Y-NH_2 interaction in tyrosinase active site.

pH value became higher than that of the free acid form. This was expected from our group's previous work.[16] To compare their storage stability, KA and KA-F-NH_2 were stored at 50 °C as solution state (20 μM, in DI water), and their inhibition activities were measured periodically. The tyrosinase inhibitory activity of KA was decreased from 18% to 8% in 3 days, however that of KA-F-NH_2 was not changed. We imagine that the aromatic side chain residues make KA-F-NH_2 be piled up and eventually lead to lower its solubility. And the lowered solubility might prevent it from fast oxidation in water. Actually, KA-F-NH_2 is less soluble than KA. As turned out in Figure 4, the tyrosinase inhibitory activity of KA was sharply decreased, whereas that of KA-F-NH_2 was well maintained even after 3 months.

As mentioned before, when amino acids having an aromatic ring structure at the side chain were conjugated to KA, its tyrosinase inhibitory activity was enhanced to over 90%. We suggest that the aromatic ring structure may contribute to the binding of the inhibitor to the hydrophobic pocket of the enzyme near the binuclear copper active site. Recently, the crystallographic structure of tyrosinase has been revealed. The three-dimensional structure of tyrosinase enables us to gain a better understanding of the tyrosinase inhibition mechanism. Although the structure of mushroom tyrosinase was not determined yet, we can borrow the crystallographic data because there is a high homology for the active center of most tyrosinase from different origin.[25–27] To ascertain the importance of the aromatic ring structure of KA-AA-NH_2, we docked KA-AA-NH_2 into the active site of tyrosinase using the AUTODOCK Tools program. According to the docking calculations, KA-AA-NH_2 having an aromatic ring structure emitted a higher free energy after docking to the active site of tyrosinase and, in this way, low energy levels were achieved.[28] We also found that quite a few hydrophobic amino acids were located around the copper active site of tyrosinase. From these data, we confirmed that there existed hydrophobic interactions between the aromatic rings of KA-F-NH_2 and the hydrophobic side chains in the tyrosinase active site, and these interactions blocked the accessibility of the substrate to the active site. For example, the aromatic side chain of KA-F-NH_2 and KA-W-NH_2 might interact with the hydrophobic pocket around His63, His216, and Phe59 surrounding the binuclear copper active site of tyrosinase. In the case of KA-Y-NH_2, the phenol ring might interact with His216, Phe66, and Trp254 (Fig. 5). Because of these interactions, we can conclude that KA-AA-NH_2, which has an aromatic ring structure at the side chain, exhibits much higher tyrosinase inhibitory activity than KA.

In summary, we prepared 20 kinds of KA-AA-NH_2 derivatives and screened their tyrosinase inhibitory activities. Among them, KA-F-NH_2, KA-Y-NH_2, KA-W-NH_2, and KA-H-NH_2 showed much higher tyrosinase inhibitory activity and their enhanced inhibitory activity was maintained for over 3 months. We also confirmed that the KA-F-NH_2 reduced the amount of dopachrome production during the melanin formation. Finally, we suggest a tyrosinase inhibition mechanism of KA-AA-NH_2 based on the possible hydrophobic interactions between the side chain of KA-AA-NH_2 and tyrosinase active site by a docking program. Further studies are on progress to confirm the tyrosinase inhibitory activity of KA-AA-NH_2 in the cell system.

Acknowledgments

This work was supported by a grant from the Korea Health Z1 R&D Project, Ministry & Welfare, Republic of Korea (A050432).

References and notes

1. Parvez, S.; Kang, M.; Chung, H.-S.; Cho, C.; Hong, M.-C.; Shin, M.-K.; Bae, H. *Phytother. Res.* **2006**, *20*, 921.
2. Parvez, S.; Kang, M.; Chung, H.-S.; Bae, H. *Phytother. Res.* **2007**, *21*, 805.
3. Kim, Y. M.; Yun, J.; Lee, C.-K.; Lee, H.; Min, K. R.; Kim, Y. *J. Biol. Chem.* **2002**, *18*, 16340.
4. Battaini, G.; Monzani, E.; Casella, L.; Lonardi, E.; Tepper, A. W. J. W.; Canters, G. W.; Bubacco, L. *J. Biol. Chem.* **2002**, *47*, 44606.
5. Kahn, V.; Ben-Shalom, N.; Zakin, V. *J. Agric. Food Chem.* **1997**, *45*, 4460.
6. Burdock, G. A.; Soni, M. G.; Carabin, I. G. *Regul. Toxicol. Pharmacol.* **2001**, *33*, 80.
7. Schurink, M.; Van Berkel, W. J. H.; Wichers, H. J.; Boeriu, C. G. *Peptides* **2007**, *28*, 485.
8. Chen, J. S.; Wei, C.-I.; Marshall, M. R. *J. Agric. Food Chem.* **1991**, *11*, 1897.
9. Marguerite, F.; Horowitz, N. H.; Heinemann, S. F. *J. Biol. Chem.* **1963**, *238*, 2045.
10. Kobayashi, Y.; Kayahara, H.; Tadasa, K.; Tanaka, H. *Bioorg. Med. Chem. Lett.* **1996**, *12*, 1303.
11. Kadokawa, J.; Nisikura, T.; Muraoka, R.; Tagaya, H.; Fukuoka, N. *Synth. Commun.* **2003**, *7*, 1081.
12. Nishimura, T.; Kometani, T.; Takii, H.; Terada, Y.; Shigetaka, O. *J. Ferment. Bioeng.* **1994**, *1*, 37.
13. O'Brien, G.; Patterson, J. M.; Meadow, J. R. *J. Org. Chem.* **1962**, *27*, 1711.
14. Kobayashi, Y.; Kayahara, H.; Tadasa, K.; Nakamura, T.; Tanaka, H. *Biosci., Biotechnol., Biochem.* **1995**, *9*, 1745.
15. Kim, H.; Choi, J.; Cho, J. K.; Kim, S. Y.; Lee, Y. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2843.
16. Noh, J. M.; Kwak, S. Y.; Kim, D. H.; Lee, Y. S. *Biopolymers* **2007**, *2*, 300.
17. Kojic acid (5 g, 35 mmol) was dissolved in THF (100 mL). After stirring for 1 h, with N_2 purging, CDI (5.1 g, 0.9 equiv) in THF (50 mL) was added, and the mixture stirred for 24 h at room temperature. A white solid powder was filtered and dried. Yield: 75%; ^{1}H NMR (DMSO-d_6, 300 MHz, δ): 9.34 (1H, s, –OH), 8.33 (1H, s, N–CH=N), 8.13 (1H, s, –CH–O), 7.67 (1H, s, imidazole), 7.10 (1H, s, imidazole), 6.66 (1H, s, CH–C=O), 5.30 (2H, s, CH_2–O). *N*-Fmoc-amino acid (2 equiv), in NMP, was quantitatively introduced to the Rink amide AM SURE® resin (0.76 mmol/g) using the general protocol of BOP-mediated solid phase Fmoc/*t*Bu strategy. After removing Fmoc group, activated kojic acid (2 equiv) was added and shaken for 6 h. Finally, the resin was treated with 30% TFA/DMC for 1 h, and filtered. The crude product in the filtrate was concentrated and precipitated with cold diethyl ether.
KA-F-NH_2 (5-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl-1-amino-1-oxo-3-phenyl-propan-2-ylcarbamate); White powder, yield: 86%, purity: 95% (HPLC; Waters μBondapak C18 reverse phase column, 125 Å, 10 μm, 3.9 × 150 mm; gradient elution with A: 0.1% TFA/water, B: 0.1% TFA/acetonitrile; from 10% to 90% B over 50 min, flow: 1 ml/min; detection; UV, 230 nm), ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.09 (1H, s, –NH), 7.65 (1H, s, =CH–O), 7.50 (1H, d, benzene), 7.24 (1H, d, benzene), 7.16 (1H, s, benzene), 6.49 (1H, s, O=CH=C), 6.26 (2H, s, NH_2), 5.06 (1H, s, NH–CH–CO), 4.77 (2H, d, –CH_2–O), 3.14 (2H, d, CH_2-benzene), ^{13}C NMR (DMSO, 300 MHz) δ 173.5 (C4), 163.5 (C2), 160.0 (C1′), 155.3 (C5), 153.5 (C2′), 145.9 (C4′), 141.0 (C6′), 140.0 (C8′), 138.2 (C6), 129.2 (C5′), 128.0 (C9′), 125.0 (C7′), 113.2 (C3), 64.9 (C1), 61.5 (C2′), 57.2 (C3′), MS (ES): $[M+1]^+$ 333.3. KA-Y-NH_2; Yield: 80%, purity: 93%, KA-W-NH_2; yield: 87%, purity: 95%, KA-S-NH_2; yield: 76%, purity: 92%, KA-T-NH_2; yield: 73%, purity: 86%, KA-D-NH_2; Yield: 83%, purity: 80%, KA-N-NH_2; yield: 79%, purity: 84%, KA-E-NH_2; yield: 80%, purity: 92%, KA-Q-NH_2; yield: 75%, purity: 93%, KA-K-NH_2; yield: 71%, purity: 89%, KA-R-NH_2; yield: 76%, purity: 95%, KA-H-NH_2; yield: 80%, purity: 96%, KA-C-NH_2; yield: 70%, purity: 70%, KA-M-NH_2; yield: 82%, purity: 92%, KA-P-NH_2; yield: 74%, purity: 86%, KA-G-NH_2; yield: 76%, purity: 96%, KA-A-NH_2; yield: 77%, purity: 93%, KA-V-NH_2; yield: 76%, purity: 89%, KA-F-NH_2; yield: 80%, purity: 91%, KA-F-NH_2; yield: 80%, purity: 90%.
18. Kermasha, S.; Goetghebeur, M.; Monfette, A.; Metche, M.; Rovel, B. *Phytochemistry* **1993**, *2*, 349.
19. Li, B.; Huang, Y.; Paskewitz, S. M. *FEBS Lett.* **2006**, *580*, 1877.
20. Park, Y. D.; Kim, S. Y.; Lyou, Y. J.; Lee, D. Y.; Yang, J. M. *Biochem. Cell Biol.* **2006**, *84*, 112.
21. Shin, N. H.; Ryu, S. Y.; Choi, E. J.; Kang, S. H.; Chang, I. M.; Min, K. R.; Kim, Y. *Biochem. Biophys. Res. Commun.* **1998**, *243*, 801.
22. Bernard, P.; Berthon, J. Y. *Int. J. Cosmet. Sci.* **2000**, *22*, 219.
23. Kim, Y. M.; Yun, J.; Lee, C. K.; Lee, H.; Min, K. R.; Kim, Y. *J. Biol. Chem.* **2002**, *18*, 16340.
24. Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. *Nat. Struct. Biol.* **1998**, *12*, 1084.
25. Steiner, R. A.; Kooter, I. M.; Dijkastra, B. *Biochemistry* **2002**, *41*, 7955.
26. Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. *J. Biol. Chem.* **2006**, *13*, 8981.
27. Naka, H.; Kondo, Y.; Usui, S.; Hashimoto, Y.; Uchiyama, M. *Adv. Synth. Catal.* **2007**, *349*, 595.
28. Khatib, S.; Nerya, O.; Musa, R.; Tamir, S.; Peter, T.; Vaya, J. *J. Med. Chem.* **2007**, *50*, 2676.