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Enzymatic formation of an aromatic dodecaketide by engineered plant polyketide synthase

Kiyofumi Wanibuchi[a,b], Hiroyuki Morita[a], Hiroshi Noguchi[b], Ikuro Abe[a,*]

[a] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] School of Pharmaceutical Sciences, University of Shizuoka and Global COE Program, Shizuoka 422-8526, Japan

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ABSTRACT

Octaketide synthase (OKS) from *Aloe arborescens* is a plant-specific type III polyketide synthase (PKS) that catalyzes iterative condensations of eight molecules of malonyl-CoA to produce the C_{16} aromatic octaketides SEK4 and SEK4b. On the basis of the crystal structures of OKS, the F66L/N222G double mutant was constructed and shown to produce an unnatural dodecaketide TW95a by sequential condensations of 12 molecules of malonyl-CoA. The C_{24} naphthophenone TW95a is a product of the minimal type II PKS (*whiE* from *Streptomyces coelicolor*), and is structurally related to the C_{20} decaketide benzophenone SEK15, the product of the OKS N222G point mutant. The C_{24} dodecaketide naphthophenone TW95a is the first and the longest polyketide scaffold generated by a structurally simple type III PKS. A homology model predicted that the active-site cavity volume of the F66L/N222G mutant is increased to 748 Å^3, from 652 Å^3 of the wild-type OKS. The structure-based engineering thus greatly expanded the catalytic repertoire of the simple type III PKS to further produce larger and more complex polyketide molecules.

The chalcone synthase (CHS) (EC 2.3.1.74) superfamily of type III polyketide synthases (PKSs) are structurally and mechanistically simple enzymes.[1–3] In contrast to the type I (modular type) and type II (subunit type) PKSs of the megaenzyme systems, the structurally simple type III PKSs accept free CoA thioesters as substrates without the involvement of an acyl carrier protein, and perform iterative condensation and cyclization reactions to produce an array of chemically and structurally divergent polyphenol scaffolds. As we previously reported, octaketide synthase (OKS) from *Aloe arborescens* is a plant-specific type III PKS that catalyzes iterative condensations of eight molecules of malonyl-CoA to yield a 1:4 mixture of the aromatic octaketides SEK4 and SEK4b (Scheme 1A and Fig. 1A).[4] The C_{16} aromatic octaketides are the products of the minimal type II PKS for the benzoisochromanequinone actinorhodin (act from *Streptomyces coelicolor*).[5,6] Furthermore, we reported that the substitution of the active-site Asn222 of OKS with Gly yielded a mutant that catalyzes the formation of the C_{20} decaketide benzophenone SEK15, by the condensation of ten molecules of malonyl-CoA (Scheme 1B and Fig. 1B).[7] The decaketide benzophenone was previously reported as a product of genetically engineered type II PKSs.[5] The octaketide-forming OKS thus gained the decaketide synthase activity by the simple steric modulation of a single, chemically inert, residue lining the active-site cavity. Indeed, the crystal structures of the wild-type and N222G mutant enzymes revealed that the large-to-small substitution increased the volume of the active-site cavity to 693 Å^3, from 652 Å^3 of the wild-type OKS (Fig. 2A and B).[1,8]

To further manipulate the OKS enzyme reaction, we expanded the polyketide elongation tunnel of the N222G mutant by simultaneously substituting the neighboring Phe66 residue, at the bottom of the tunnel, with less bulky amino acids. Thus, we constructed a set of *A. arborescens* OKS double mutants (F66G/N222G, F66A/N222G, F66V/N222G, F66S/N222G, and F66L/N222G), and investigated the mechanistic consequences of the mutations.[9] A homology model based on the N222G mutant predicted that the active-site cavity volume is increased to 748 Å^{3}in the F66L/N222G double mutant (Fig. 2C).

The double mutants were heterologously expressed in *Escherichia coli* as His-tagged fusion proteins, at similar levels to the wild-type enzyme, and were purified to homogeneity, as previously reported for the wild-type and N222G mutant enzymes.[10] When incubated with malonyl-CoA as a substrate, the F66V/N222G and F66S/N222G mutants displayed enzyme activities similar to that of the N222G point mutant, and efficiently produced the decaketide benzophenone SEK15, whereas the F66G/N222G and F66A/N222G mutants completely lost the enzyme activity.[11] Notably, the OKS F66G point mutant also lacked the enzyme activity, and the F66A mutant showed a notable decrease of the activity. These results suggested that the substitution of Phe66 with the

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Abbreviations: OKS, Octaketide synthase; PCS, Pentaketide chromone synthase; CHS, Chalcone synthase; PKS, Polyketide synthase.

[*] Corresponding author. Tel.: +81 3 5841 4740; fax: +81 3 5841 4744.
E-mail address: abei@mol.f.u-tokyo.ac.jp (I. Abe).

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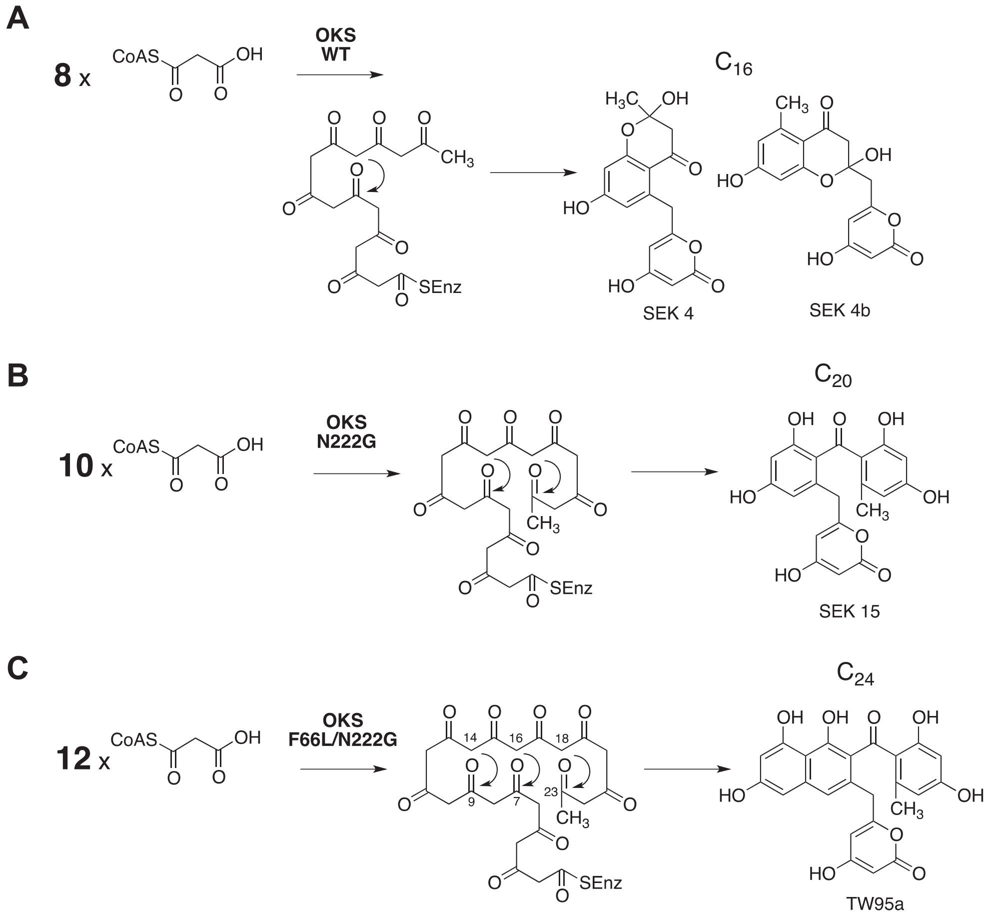

Scheme 1. Proposed mechanism for the formation of (A) SEK4 and SEK4b by the wild-type OKS, (B) SEK 15 by the N222G mutant OKS, and (C) TW95a by the F66L/N222G mutant OKS.

small Gly or Ala residue causes significant conformational changes in the folding of the enzyme, which lead to the loss of the enzyme activity.

On the other hand, the major product generated by the F66L/N222G double mutant was obtained in 6% yield (calculated by an incubation with [2-^{14}C]malonyl-CoA[12] as the substrate under the standard assay conditions), in addition to the decaketide SEK15 as a minor product (Fig. 1C). The major product gave a UV spectrum (λ_{max} 234, 284, 340, and 402 nm) and a parent ion peak $[M+H]^+$ at m/z 451 on LC-ESIMS, indicating the formation of a product of 12 condensations of malonyl-CoA. The spectroscopic data (LC-ESIMS, UV, and ^{1}H NMR) of the product obtained from a large scale incubation were quite similar to those of the C_{20} benzophenone SEK15, with the 4-hydroxy-2-pyrone and 2,4-hydroxy-6-methylphenyl ketone functionalities.[13] However, the ^{1}H NMR spectrum revealed the presence of five aromatic protons (δ 7.01, 6.59, 6.40, 6.18, and 6.14), indicating the presence of a naphthophenone, instead of the benzophenone moiety. Thus, the spectroscopic data were identical to those of the previously reported C_{24} dodecaketide naphthophenone TW95a, which is a product of the minimal type II PKS (*whiE* from *S. coelicolor*),[14] and is structurally related to the C_{20} decaketide benzophenone SEK15, the product of the OKS N222G point mutant.[7] This is the first demonstration of C_{24} dodecaketide production by a structurally simple type III PKS.

A steady-state kinetics analysis revealed a $K_M = 32\ \mu M$ and a $k_{cat} = 4.7 \times 10^{-2}\ min^{-1}$ for malonyl-CoA by the OKS F66L/N222G double mutant with respect to the TW95a-forming activity, with a pH optimum at 6.5.[15] On the other hand, the previously reported SEK15-forming OKS N222G mutant exhibited a $K_M = 55\ \mu M$ and a $k_{cat} = 2.7 \times 10^{-3}\ min^{-1}$, and the SEK4b-forming wild-type OKS showed a $K_M = 94\ \mu M$ and a $k_{cat} = 9.4 \times 10^{-2}\ min^{-1}$. Notably, the enzyme activities of the N222G and F66L/N222G mutants are comparable to that of the wild-type OKS, and the K_M values suggest even higher affinity for the substrate binding to the active-site, despite the lower k_{cat} values.

It is remarkable that the OKS F66L/N222G mutant not only catalyzed the condensation of 12 molecules of malonyl-CoA but also attained the naphthalene ring forming activity to produce the C_{24} dodecaketide naphthophenone TW95a, which is the first and the longest polyketide scaffold generated by a structurally simple type III PKS. As mentioned above, a homology model predicted that the active-site cavity volume of the dodecaketide-producing N222G/F66L double mutant is increased to 748 Å^3 from 693 Å^3 of the decaketide-producing N222G point mutant, and from 652 Å^3 of the octaketide-producing wild-type OKS. Thus, the replacement of the two residues, Phe66 and Asn222, expanded the active-site cavity, thus extending the number of condensation reactions and the resulting polyketide chain length. It is remarkable that the functional conversion appeared to be caused by the simple steric modulation of the active-site, accompanied by the conservation of the Cys-His-Asn catalytic triad. This was also the case for the previously reported *A. arborescens* pentaketide chromone synthase (PCS), which produces 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA.[16,17] The structure-based PCS F80A/Y82A/M207G triple mutant, with an expanded active-site cavity,

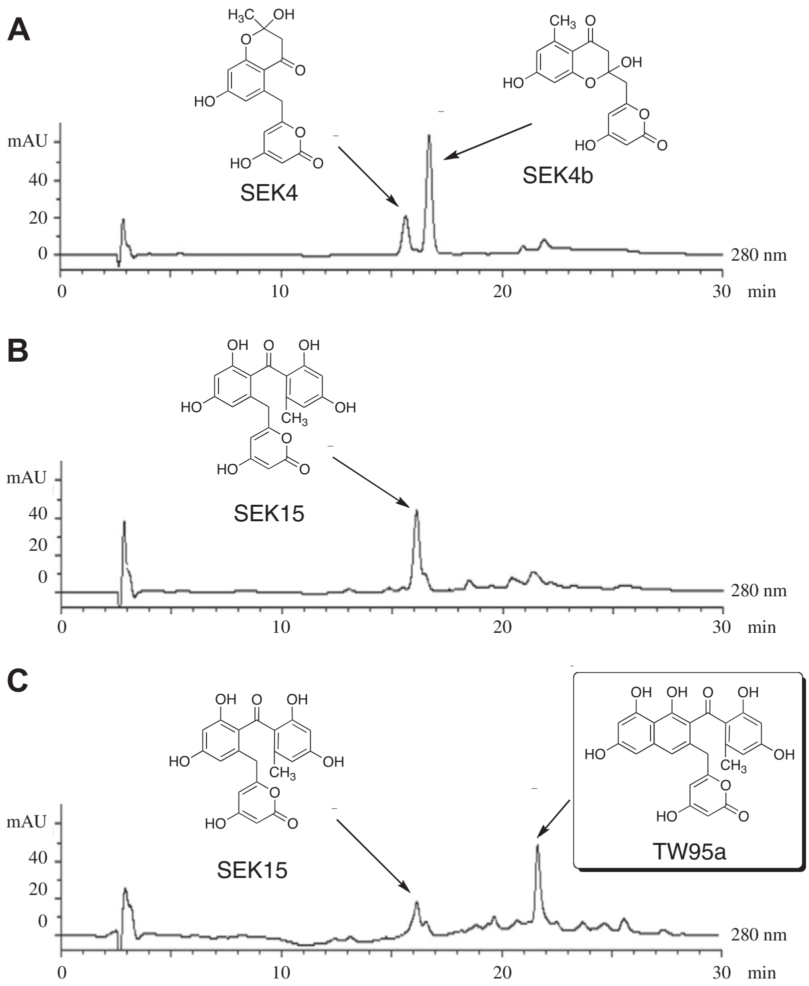

Figure 1. HPLC elution profiles of the enzyme reaction products of (A) the wild-type OKS, (B) the N222G mutant OKS, and (C) the F66L/N222G mutant OKS.

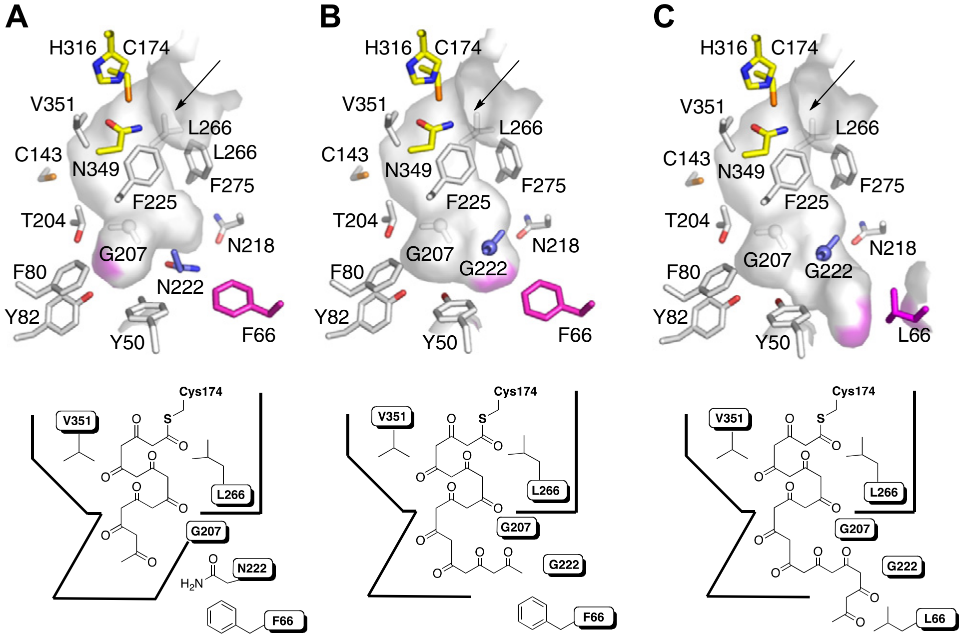

Figure 2. Surface and schematic representations of the active-site architectures of the wild-type and mutant OKSs. Crystal structure of (A) the wild-type OKS, and (B) the N222G mutant OKS, and homology model of (C) the F66L/N222G mutant OKS. The substrate entrances are indicated with arrows. The bottoms of the active-site are indicated by purple surfaces. The Cys-His-Asn catalytic triads are shown as yellow stick models. The mutated residues Asn222 (Gly222) and Phe66 (Leu66) are highlighted as blue and purple stick models, respectively.

was shown to produce the unnatural novel nonaketide naphthopyrone by sequential condensations of nine molecules of malonyl-CoA.[18]

The proposed mechanism for the formation of the dodecaketide naphthophenone TW95a by the F66L/N222G mutant closely parallels the case of the decaketide SEK15 produced by the N222G mutant, and involves consecutive intramolecular aldol-type condensations and terminal α-pyrone ring formation (Scheme 1B and C). Presumably, the enzyme catalyzes the first aromatic ring formation reaction at the middle of the polyketide intermediate during the sequential decarboxylative condensations of 12 molecules of malonyl-CoA. Thus, dual C–C bond formation at C-16/C-7 and C-14/C-9 generates the naphthalene ring system of the dodecaketide TW95a, whereas a single aldol-type cyclization produces the benzene ring of the decaketide SEK15. Alternatively, the aromatic ring formation is possibly initiated at the methyl end of the elongating polyketide intermediate, as in the case for the formation of the octaketide SEK4b by the wild-type OKS. The partially cyclized aromatic intermediates would then be released from the active-site by the formation of the terminal α-pyrone ring, which could be an important process for the release of the polyketide products from the thioester-linked active-site Cys residue. Finally, it should be noted that the formation of the dodecaketide naphthophenone by the *A. arborescens* OKS mutant suggests further involvement of the CHS-superfamily type III PKSs in the biogenesis of the anthrone and anthraquinone scaffolds in the aloe plant.[4]

In summary, this is the first demonstration of the formation of a dodecaketide by a structurally simple type III PKS. The structure-based engineering thus greatly expanded the catalytic repertoire of the simple CHS superfamily type III PKS, to produce larger, more complex polyketide molecules.

Acknowledgments

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9. *Site-directed mutagenesis:*The plasmids expressing *Aloe arborescens* OKS double mutants (F66G/N222G, F66A/N222G, F66V/N222G, F66S/N222G, and F66L/N222G) were constructed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol, using the following pairs of primers (mutated codons are underlined) and the previously reported plasmid bearing the N222G OKS mutant as the template: F66G (5′-GGTCGAGCTCAAGAAGAAGGGCGATCGCAT-3′) and (5′-TGTCTTTTTGCAGATGCGATCGCCCTTCTTCTTGAG-3′), F66A (5′-GGTCGAGCTCAAGAAGAAGGCCGATCGCAT-3′ and 5′-TGTCTTTTTGCAGATGCGATCGGCCTTCTTCTTGAG-3′), F66V (5′-GGTCGAGCTCAAGAAGAAGGTCGATCGCAT-3′) and (5′-TGTCTTTTTGCAGATGCGATCGACCTTCTTCTTGAG-3′), F66S (5′-GGTCGAGCTCAAGAAGAAGTCGGATCGCAT-3′) and (5′-TGTCTTTTTGCAGATGCGATCCGACTTCTTCTTGAG-3′), and F66L (5′-GGTCGAGCTCAAGAAGAAGTTGGATCGCAT-3′ and 5′-TGTCTTTTTGCAGATGCGATCCAACTTCTTCTTGAG-3′).
10. *Expression and purification:* After confirmation of the sequence, the plasmid was transformed into *E. coli* BL21(DE3)pLysS. The cells harboring the plasmid were cultured to an A_{600} of 0.6 in LB medium containing 100 μg/mL ampicillin at 37 °C. Subsequently, 1.0 mM isopropylthio-β-D-alactopyranoside was added to induce protein expression, and the cells were further cultured at 23 °C for 16 h. All of the following procedures were performed at 4 °C. The *E. coli* BL21(DE3)pLysS cells transformed with the OKS mutants were harvested by centrifugation and resuspended in 40 mM potassium phosphate buffer, pH 7.9, containing 0.1 M NaCl and 5 mM imidazole. The cells were disrupted by sonication and centrifuged at 10,000*g* for 30 min. The supernatant was passed through a Ni Sepharose™ 6 Fast Flow column (GE Healthcare). After the column was washed with 20 mM potassium phosphate buffer, pH 7.9, containing 0.5 M NaCl and 40 mM imidazole, the recombinant enzyme was finally eluted with 15 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol and 500 mM imidazole. The recombinant PKSs were fairly soluble, and the Ni-chelate affinity column chromatography afforded 3–5 mg of pure enzymes from 1 L of the *E. coli* culture. The protein concentration was determined by the Bradford method (Protein Assay Dc, Bio-Rad, Hercules, CA, USA) using bovine gamma globulin as the standard.
11. *Enzyme reaction:* The standard reaction mixture contained 216 nmol of malonyl-CoA and 10 μg of the purified recombinant enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 6.5. Reactions were incubated at 30 °C for 16 h. The products were then extracted with 2 mL of ethyl acetate. The products were separated by reverse-phase HPLC (JASCO 880) on a TSK-gel ODS-80Ts column (4.6 × 150 mm, TOSOH), at a flow rate of 0.8 mL/min. Gradient elution was performed with H_2O and MeOH, both containing 0.1% TFA: 0–5 min, 30% MeOH; 5–17 min, linear gradient from 30% to 60% MeOH; 17–25 min, 60% MeOH; 25–27 min, linear gradient from 60% to 70% MeOH. Elutions were monitored by a multichannel UV detector (MULTI 340, JASCO) at 280 nm; UV spectra (200–400 nm) were recorded every 0.4 s. Online LC-ESIMS spectra were measured with an Agilent Technologies HPLC 1100 series HPLC coupled to a Bruker Daltonics Esquire4000 ion trap mass spectrometer fitted with an ESI source. HPLC separations were performed under the same conditions as described above. The ESI capillary temperature and the capillary voltage were 320 °C and 4.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the positive mode, over a mass range of 50–600 *m*/*z*, and at a range of one scan every 0.2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV). The samples for the LC-HRMS were analyzed with an Agilent 1100 series HPLC-microTOF mass spectrometer (Bruker Daltonics) using Electrospray Ionization.
12. [2-^{14}C]Malonyl-CoA was purchased from GE Healthcare.
13. ^{1}H NMR (800 MHz, DMSO-d_6) δ 7.01 (s, 1H), 6.59 (d, 1H), 6.40 (d, 1H), 6.18 (s, 1H), 6.14 (s, 1H), 5.70 (s, 1H), 5.18 (d, 1H), 3.67 (s, 2H), 1.92 (s, 3H); UV λ_{max} 234, 284, 340 and 402 nm; ESI-MS: *m*/*z* 451 [M+H]$^+$; ESI-MS/MS: 327, 261, 191, 151; HRMS (TOF) found for $[C_{24}H_{19}O_9]^+$ 451.1006, calcd 451.1007. The data were identical to those previously reported in the literature.[14]
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15. *Enzyme kinetics*. Steady-state kinetic parameters were determined by using [2-^{14}C]malonyl-CoA (1.8 mCi/mmol) as the substrate. The experiments were performed in triplicate using five concentrations of malonyl-CoA (108, 43.2, 21.6, 10.8 and 4.3 μM) in the assay mixture, containing 108 μM of malonyl-CoA, 3 μg of purified enzyme, and 1 mM EDTA, in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 6.5. The reactions were incubated at 30 °C for 120 min. The reaction products were extracted and separated by TLC (Merck Art. 1.11798 Silica gel 60 F254; ethyl acetate/hexane/AcOH = 63:27:5, *v*/*v*/*v*). Radioactivities were quantified by autoradiography using a bioimaging analyzer BAS-2000II (FUJIFILM). Lineweaver–Burk plots of data were employed to derive the apparent K_M and k_{cat} values (average of triplicates), using the ENZFITTER software (BIOSOFT).
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