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ARTICLE

Isolation and structure determination of a new cytotoxic peptide, curacozole, from *Streptomyces curacoi* based on genome mining

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Abstract

Using genome mining, a new cytotoxic peptide named curacozole was isolated from *Streptomyces curacoi*. Through ESI-MS and NMR analyses, curacozole was determined to be a macrocyclic peptide containing two isoleucine, two thiazole and three oxazole moieties. Curacozole exhibited potent cytotoxic activity against HCT116 and HOS cancer cells. The proposed biosynthetic gene cluster of curacozole was identified and compared with that of the related compound YM-216391.

Introduction

A large collection of bacterial genome sequences have been accumulated in a database, and genome mining has been used to find new secondary metabolites by utilizing the genomic sequence data [1–3]. The biosynthetic gene clusters (BGCs) of ribosomally synthesized and post-translationally modified peptides (RiPPs), including lantibiotics, lasso peptides, and thiopeptides, are suitable targets for identification by the genome mining method since the structural prediction of the final products is comparatively easier than that for peptides biosynthesized by nonribosomal peptide synthetases (NRPSs). The prediction software antiSMASH was developed to search for secondary metabolite BGCs in whole genome sequences [4]. An increase in the understanding the biosynthetic systems of natural products has improved the ability to predict the possible biosynthetic gene clusters for secondary metabolites such as polyketides, NRPs, and RiPPs from the genomic data of bacteria and fungi [5–8]. Recently, a new prediction system for RiPPs named RODEO (Rapid ORF Description and Evaluation Online) was developed by combining the hidden-Markov-model-based analysis, heuristic scoring, and machine learning to identify biosynthetic gene clusters and predict RiPP precursor peptides [9]. This approach enabled the comprehensive mapping of RiPP biosynthetic gene clusters, revealing the existence of more than 1,300 putative compounds. Based on this bioinformatic approach, six new lasso peptides were discovered and characterized, indicating that the RODEO system is efficient [9].

Among RiPPs, telomestatin, a macrocyclic compound containing seven oxazoles and one thiazole, was isolated from *Streptomyces anulatus* as a potent telomerase inhibitor [10]. Due to its structural characteristics, telomestatin interacts with human telomeric intramolecular G-quadruplexes to form basket-type G-quadruplex (G4) structures from hybrid-type G-quadruplexes [11]. The telomestatin BGC was previously identified by analyzing the genome of *Streptomyces anulatus* 3533-SV4, which indicated that biosynthesis of telomestatin belonged to the RiPP biosynthetic system [12]. The analogous macrocyclic

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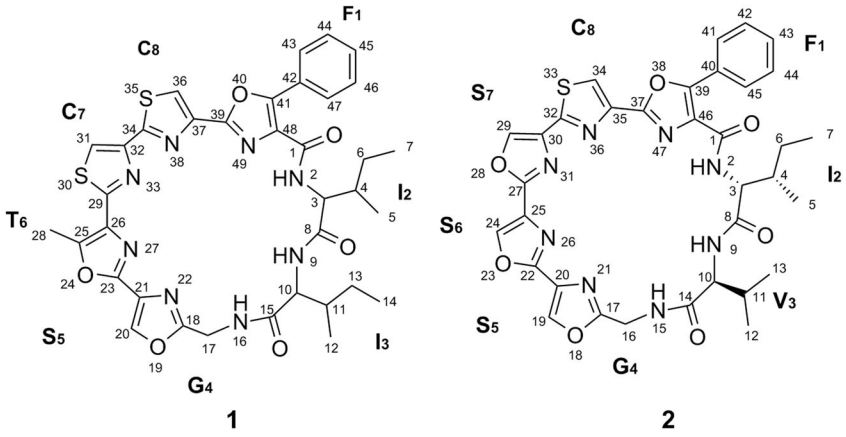

Fig. 1 Chemical structure of **1** and **2**, bold letters around compounds indicate precursor amino acids (FIIGSTCC for **1** and FIVGSSSC for **2**)

S. nobilis (YM-216391)	M-----TAEIEEVDIEVGFIVGSSCSLELEEDDLDVAADE
S. curacoi (curacozole)	MFENT-TAEIEEVDIEVGFIIGSTCCSLEMEEDDLDVAAEETEAV
S. viridochromogenes	MFENT-TAEIEEVDIEVGFIIGSTCCSLEMEEDDLDVAAEETETV
S. aurantiacus	MSENITTAEIEEVDIEVGFIIGSSCCSLEMEEDDLDVAADE

Fig. 2 Amino acid sequences of precursor peptide-coding genes for **1** and **2**, bold letters indicate conserved amino acids. Underlines indicate core peptide regions. Accession number of each gene is following *Streptomyces nobilis*: AFJ68074.1, *S. curacoi*: WP_107116988.1, *S. viridochromogenes*: WP_003997107.1, *S. aurantiacus*: WP_016640788.1

compound, YM-216391 (**2** in Fig. 1), was isolated as a cytotoxic compound from *Streptomyces nobilis* [13, 14]. The BGC of YM-216391 (**2** in Fig. 1) was determined from the genomic sequence data of *S. nobilis* JCM 4274 [15]. The gene cluster consists of a precursor peptide coding gene and modification genes, which typically compose RiPP BGCs.

Recently, the antibacterial peptide curacomycin was isolated from *Streptomyces curacoi* via genome mining [16]. By further mining the genome of *S. curacoi*, we found a gene cluster analogous to that of YM-216391 (**2** in Fig. 1). As a result of our investigation, a new cytotoxic compound named curacozole (**1** in Fig. 1) was isolated from *S. curacoi*, and its structure was determined by ESI-MS and NMR experiments. Here, we describe the isolation and structure determination of curacozole (**1**).

Results and discussion

The cytotoxic compound YM-216391 (**2** in Fig. 1) was reported to be biosynthesized from the precursor peptide (AFJ68074.1) via several enzymatic modifications [15]. Through modification, the amino acids Ser and Cys in the core peptide sequence FIVGSSSC (bold letters in Fig. 1 and underlined letters in Fig. 2) are converted to oxazoles and thiazoles. By performing a BLASTp search [17] using the amino acid sequence of the precursor peptide of YM-216391 (AFJ68074.1) [15], we found similar precursor peptide coding genes (Fig. 2) in the genomes of *S. curacoi* DSM 40107^T and *Streptomyces viridochromogenes* Tü57 [3], along with another unique precursor peptide gene in the *Streptomyces aurantiacus* JA 4570 genome. The new peptide was expected to possess the structure depicted in Fig. 1 as **1**, and it may be biosynthesized from the core peptide sequence FIIGSTCC (bold letters in Fig. 1 and underlined letters in Fig. 2). We performed HPLC and ESI-MS investigations on a MeOH extract of *S. curacoi* NBRC 12761^T cells to detect the expected peptide (**1**) shown in Fig. 1. The expected peptide was detected by the combination of HPLC and ESI-MS (data not shown).

To increase the yield of **1** in *S. curacoi*, rifampicin was used according to the previously reported method [18]. *Streptomyces* strains with a specific RNA polymerase β gene (*rpoB*) mutation that confers rifampicin resistance can produce abundant quantities of secondary metabolites [19, 20]. Briefly, the rifampicin-resistant (rif^r) *S. curacoi* mutants were obtained for curacozole production screening. Then, cell extracts of the rif^r mutants (30 strains) were analyzed by HPLC to evaluate curacozole production. We found that three rifampicin-resistant mutants harboring a 1298 C > T (Ser433Leu: S433L) mutation in the gene encoding the RNA polymerase β subunit showed a productions of **1** 3–5 times higher compared with that of the wild type (data not shown). In this study, we found that the curacozole-overproducing rif^r *S. curacoi* mutants all carried an S433L mutation in the *rpoB* gene. Previous studies showed that a *Streptomyces lividans* strain with a single S433L mutation in the *rpoB* gene overproduced the blue-pigmented antibiotic actinorhodin [21]. These findings indicate that the *rpoB* S433L mutation

effectively improves secondary metabolite production in a variety of streptomycetes. To produce **1**, we cultured a rif^r mutant designated R25.

To obtain a sufficient quantity of **1** for structure determination, *S. curacoi* R25 was cultured in 2 L of ISP2 agar media [22]. After 7 days of cultivation, spore and aerial hyphae cells were harvested and extracted with MeOH and then centrifuged to remove any insoluble compounds. The extract was concentrated by rotary evaporation and subjected to a CHP-20P column eluted with 10%, 60%, and 100% MeOH. The 100% MeOH fraction was repeatedly subjected to HPLC purification to afford **1**.

The molecular formula of **1** was established to be $C_{36}H_{36}N_8O_6S_2$ by accurate mass analysis since the ion corresponding to $[M+H]^+$ (the calculated *m/z* value, 741.2277) was observed at *m/z* 741.2284. To obtain further information on the chemical structure, NMR experiments including ^1H, ^{13}C, DEPT-135, DQF-COSY, HSQC, TOCSY and HMBC analyses of **1** were performed using DMSO-d_6 as the solvent (Table 1 and Fig. 3). The ^1H NMR spectrum of **1** exhibited 34 proton signals, including three amide protons, one aminomethylene, and two aminomethines. The HSQC experiment direct ^1H-^{13}C connections. By analyzing the TOCSY and HMBC spectra, the structure of **1** was established as shown in Fig. 3. Since **1** was expected to be an analog of YM-216391, their NMR spectral data were compared. As shown in Table 1, the chemical shift values of **1** were similar to those of **2**. There are three major differences in the NMR spectrum of **1** compared with that of **2**. The characteristic chemical shift of position 31 (δ*H* 8.60, δ*C* 120.8) indicated the presence of a thiazole group as opposed to an oxazole group. HMBC correlations from the methyl residue (H28) to C25, C26, C29 and C23 were observed, which indicated that the methyl is attached to a carbon (C25). The H-H spin system constructed by TOCSY and HMBC correlations from H14 to C13 and C11 indicated the presence of an Ile containing C14. The connections among C39, C41, and C48 were not established due to the absence of an HMBC correlation; however, the similarity of the chemical shifts (Table 1) indicated the presence of an oxazole group.

The absolute stereochemistries of the two Ile groups were analyzed by the modified Marfey's method [23], and chiral HPLC analysis was performed on the acid hydrolysate of **1**. The modified Marfey's analysis revealed the presence of L-Ile and that the mixture contained D-Ile and D-allo-Ile in a 1:1 molar ratio. The D-Ile and D-allo-Ile moieties could not be distinguished due to their identical HPLC retention times in the modified Marfey's analysis. To determine the proportions of D-Ile and D-allo-Ile, chiral HPLC analysis was performed on the hydrolysate of **1** according to the previous report [14]. The analysis revealed the presence of D-allo-Ile and D-Ile in a 6:4 molar ratio.

Table 1 NMR chemical shift values of curacozole (**1**) and YM-216391 (**2**) in DMSO-d_6

Curacozole (1)			YM-216391(2)[a]		
Position	δ*H* (*J* = Hz)	δ*C*	Position	δ*H* (*J* = Hz)	δ*C*
1		160.3	1		160.3
2	8.10, 1H (d, 7.7)		2	8.22, 1H (d, 6.5)	
3	4.78, 1H (dd, 7.7, 4.2)	56.5	3	4.82, 1H (dd, 6.5, 3.5)	57.1
4	1.94, 1H (m)	39.2	4	2.09, 1H (m)	38.8
5	0.87, 3H (ov*)	14.8	5	0.95, 3H (d, 7.5〉	14.7
6	1.57, 1H (m)	25.8	6	1.66, 1H (m)	25.6
	1.03, 1H (m)			1.09, 1H (m)	
7	0.87, 3H (ov*)	12.2	7	0.91, 3H (t, 7.5〉	12.1
8		170.2	8		170.2
9	8.53, 1H (d, 8.5)		9	8.57, 1H (d, 9.0)	
10	4.32, 1H (dd, 8.5, 6.5)	57.4	10	4.60, 1H (dd, 9.0, 4.5)	57.5
11	1.84, 1H (m)	37.0	11	2.14, 1H (m)	31.5
12	0.86, 3H (ov*)	16.0	12	0.97, 3H (d, 6.5)	17.4
13	1.23, 1H (m)	24.3	13	0.93, 3H (d, 6.5)	19.7
	1.49, 1H (m)				
14	0.81, 3H (t, 7.3)	11.0			
15		171.2	14		170.8
16	8.71, 1H (dd, 9.1, 2.5)		15	8.67, 1H (dd, 9.0, 2.0)	
17	4.99, 1H (dd, 16.6, 9.1)	35.9	16	5.05, 1H (dd, 17.0, 9.0)	35.2
	4.12, 1H (dd, 16.6, 2.3)			4.19, 1H (dd, 16.5, 2.5)	
18		162.9	17		163.0
20	8.79, 1H (s)	139.5	19	8.89, 1H (s)	139.6
21		129.6	20		129.1
23		153.4	22		155.5
25		149.1	24	8.98, 1H (s)	139.1
26		131.4	25		129.9
28	2.69, 3H (s)	11.7			
29		157.6	27		155.0
31	8.60, 1H (s)	120.8	29	9.07, 1H (s)	139.4
32		148.0	30		135.6
34		161.5	32		157.5
36	8.65, 1H (s)	123.1	34	8.65, 1H (s)	122.2
37		142.1	35		141.5
39		154.3	37		154.1
41		150.9	39		150.6
42		126.7	40		126.7

Table 1 (continued)

Curacozole (1)			YM-216391(2)[a]		
Position	δ*H* (*J* = Hz)	δ*C*	Position	δ*H* (*J* = Hz)	δ*C*
43/47	8.33, 1H (d, 7.5)	127.8	41/45	8.35, 1H (d, 7.5〉	127.5
44/46	7.55, 1H (t, 7.5)	128.8	42/44	7.57, 1H (t, 7.0)	128.6
45	7.50, 1H (t, 7.5)	130.3	43	7.52, 1H (t, 7.0)	130.0
48		130.6	46		130.8

*ov** overlapped

[a]Chemical shift values of **2** was excerpted from ref. [14]

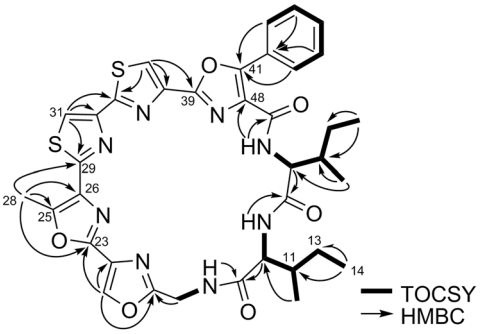

Fig. 3 Selected 2D NMR correlations of **1**

To investigate whether treatment with **1** alters the proliferation of cancer cells, we incubated HCT116 and HOS cells with various concentrations of **1** for 72 h and assessed cell viability by the CellTiter-Glo luminescent cell viability assay. Treatment with **1** resulted in a dose-dependent cytotoxicity in these cell lines and exhibited IC_{50} values of 8.6 nM and 10.5 nM for HCT116 and HOS cells, respectively (data not shown). These data indicate that **1** is highly toxic towards HCT116 and HOS cancer cells.

In this study, we found a new precursor peptide gene similar to that of YM-216391 in the *S. curacoi* genome by genome mining (Fig. 2). We successfully isolated the product, named curacozole (**1**), from the strain. Thus, the cluster containing the precursor peptide gene was considered to be responsible for the synthesis of **1**. This gene cluster includes ten genes (Fig. 4) and shares a similar gene organization to that of YM-216391 [15]. Nine of the ten genes, with the exception of AQI70_RS14565, are orthologues of *ymI, ymA, ymD, ymE, ymB1, ymC1, ymF, ymBC* and *ymR1*, genes responsible for the biosynthesis of YM-216391 [15]. The translated amino acid sequences of the genes showed 69 to 97% similarity to Ym proteins (Table S1). In light of the structural similarity between **1** and **2** (Fig. 1) and the YM-216391-biosynthetic pathway [15], we propose the biosynthetic pathway of **1** (Fig. 5). A linear precursor peptide of 44 amino acid residues (AQI70_RS38515, Fig. 2) is ribosomally synthesized. A docking protein (AQI70_RS14600) and a cyclodehydratase-oxidoreductase didomain enzyme (AQI70_RS37415) convert G-S-T-C-C into the Gly-oxazole-methyloxazole-thiazole-thiazole moiety. The P-450 (AQI70_RS14595) hydroxylates the Phe residue in the intermediate peptide. The protease (AQI70_RS14580) cleaves the core peptide, which corresponds to **1**, at both the N- and C-termini of the full-length precursor, which is followed by N-C terminal cyclization. This cyclic peptide formation may require the assistance of a hypothetical protein (AQI70_RS14605). The cyclodehydratase (AQI70_RS14590) cyclizes thiazole-β-hydroxy-Phe, and then the FAD-binding monooxygenase (AQI70_RS14585) oxidizes the ring to form a thiazole-phenyloxyazole moiety. Although, one of the two Ile residues in **1** may be D-Ile/D-*allo*-Ile, as this cluster does not include an epimerase homologous to YmG. The epimerase responsible for the conversion of L-Ile into D-Ile/D-*allo*-Ile still remains to be identified. In cypemycin biosynthesis, CypI is proposed to isomerize L-Ile residues [24]. The S. *curacoi* genome encodes a homolog of CypI (AQI70_17525) at a locus distant from the cluster, which may catalyze the isomerization.

Recently, genome mining has often been employed to search for RiPP compounds such as lantibiotics, lasso peptides and thiopeptides [25, 26]. Nevertheless, to the best of our knowledge, this is the first report on the genome mining-based discovery of a new sequential oxazole/methyloxazole/thiazole ring-containing macrocyclic peptide from actinomycetes. This may be due to the fact that few compounds of this class of peptides have been reported to date [12, 15]. Investigation of these compounds and their BGCs will contribute to the acceleration of secondary metabolite discovery in the post-genomic era.

Materials and methods

Microbial strains

The bacterial strain *Streptomyces curacoi* NBRC 12761^T was obtained from the NBRC culture collection (NITE Biological Resource Center, Japan). Spontaneous rifampicin-resistant (rif^r) mutants of *S. curacoi* NBRC 12761 were obtained from colonies grown within 10 days after the spores or hyphal fragments (approximately 2×10^9 colony forming units) were spread on GYM agar medium [27] containing 10 μg/mL of rifampicin, which corresponds to an amount that is 2-fold greater than the minimum inhibitory concentration.

Mutation analysis of the *rpoB* gene

The partial *rpoB* gene fragment in rif^r mutants was obtained using the primers 5′-GGCGCTCGGCTGGACGACCG-3′ (forward) and 5′-CGATCAGACCGATGTTCGGG-3′ (reverse), which were designed based on the sequence in

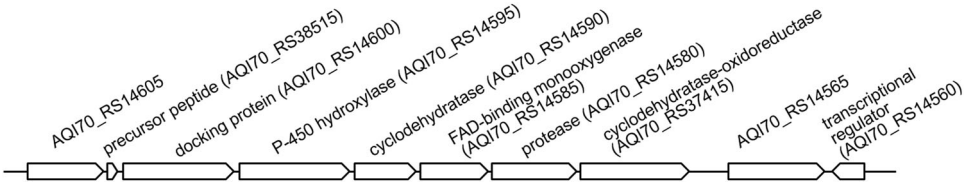

Fig. 4 Biosynthetic gene cluster for curacozole (**1**). The genes AQI70_RS14605, AQI70_RS38515, AQI70_RS14600, AQI70_RS14595, AQI70_RS14590, AQI70_RS14585, AQI70_RS14580, AQI70_RS37415 and AQI70_RS14560 encode orthologues for *ymI, ymA, ymD, ymE, ymB1, ymC1, ymF, ymBC* and *ymR1*, respectively. Accession numbers of the genes are following; AQI70_RS14605: WP_062148887, AQI70_RS38515:WP_107116988, AQI70_RS14600: WP_079051299, AQI70_RS14580:WP_062148872, AQI70_RS37415: WP_062148869, AQI70_RS14565:WP_062148866, AQI70_RS14560: WP_062148863.1

S. curacoi NBRC 12761^{T}. PCR amplification was carried out with Tks Gflex DNA polymerase (TaKaRa Bio, Inc., Shiga, Japan). The purified PCR product was subjected to DNA sequence analysis, which was performed by Eurofins Genomics K. K. (Tokyo, Japan). The sequence data were aligned using the ClustalW program at DDBJ (http://clustalw.ddbj.nig.ac.jp/).

Isolation of curacozole

The *Streptomyces curacoi* mutant strain R25 was cultured in 2 L of ISP2 agar medium [22] at 30 °C for 7 days. The aerial hyphae and spore cells were harvested and then extracted with MeOH. Approximately 200 ml of MeOH was added to the harvested cells, and the extract was filtered through filter paper (Whatman No.1, GE Healthcare Life Sciences, Little Chalfont, UK). The extract was concentrated to an aqueous residue by a rotary evaporator. The concentrated extract was subjected to open column chromatography (styrene-divinylbenzene resin, CHP-20P, Mitsubishi Chemical Corp., Tokyo, Japan) eluted with 10% MeOH, 60% MeOH and 100% MeOH. The 100% MeOH fraction was concentrated on a rotary evaporator and then separated via HPLC using an ODS column (4.6 × 250 mm, Wakopak Handy ODS, WAKO). The UV detector of HPLC was set at a wavelength of 220 nm. 100% MeOH fraction was subjected to HPLC with elution of 73% MeCN containing 0.05% TFA (trifluoroacetic acid) at flow rate of 1 mL/min to yield 4.0 mg of curacozole (retention time; 12.0 min).

MS experiments

ESI-MS analyses were performed using a JEOL JMS-T100LP mass spectrometer. For accurate MS analysis, reserpine was used as an internal standard.

NMR experiments

An NMR sample was prepared by dissolving **1** in 500 µl of DMSO-d_6. All NMR spectra were obtained on Bruker Avance 600 and Avance III HD 800 spectrometers with quadrature detection in the phase-sensitive mode by States-TPPI (time-proportional phase incrementation) and in the echo-antiecho mode. One-dimensional (1D) ^{1}H, ^{13}C, and DEPT-135 spectra were recorded at 25 °C with a 12 ppm window for proton and 239 ppm or 222 ppm windows for carbon. The following 2D ^{1}H-NMR spectra were recorded at 25 °C with 12 ppm or 15 ppm spectral widths in the t1 and t2 dimensions: 2D double-quantum filtered correlated spectroscopy (DQF-COSY), recorded with 512 and 1024 complex points in the t1 and t2 dimensions; 2D homonuclear total correlated spectroscopy (TOCSY) with MLEV-17 mixing sequence, recorded with mixing time of 80 ms, 256 and 1024 complex points in t1 and t2 dimensions; 2D nuclear Overhauser effect spectroscopy (NOESY), recorded with mixing time of 200 and 400 ms, 256 and 1024 complex points in the t1 and t2 dimensions. 2D ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond connectivity (HMBC) spectra were acquired at 25 °C in the echo-antiecho mode. The ^{1}H-^{13}C HSQC and HMBC spectra were recorded with 1024 × 512 complex points for 12 ppm in the ^{1}H dimension and 160 ppm or 222 ppm in the ^{13}C dimension, respectively, at a natural isotope abundance.

All NMR spectra were processed using TOPSPIN 3.5 (Bruker). Before Fourier transformation, the shifted sinebell window function was applied to the t1 and t2 dimensions. All ^{1}H and ^{13}C dimensions were referenced to DMSO-d_6 at 25 °C.

Modified Marfey's analysis

Compound **1** was subjected to acid hydrolysis with 6N HCl at 110 °C for 16 h. The hydrolysate was concentrated to dryness using a rotary evaporator, and then 200 µL of water was added. To the hydrolysate, 10 µL of a solution of *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA, Tokyo Chemical Industry Co., LTD, Tokyo, Japan) in acetone (10 µg/µL) and 100 µL of 1 M $NaHCO_3$ solution were added,and the mixture was incubated at 80 °C for

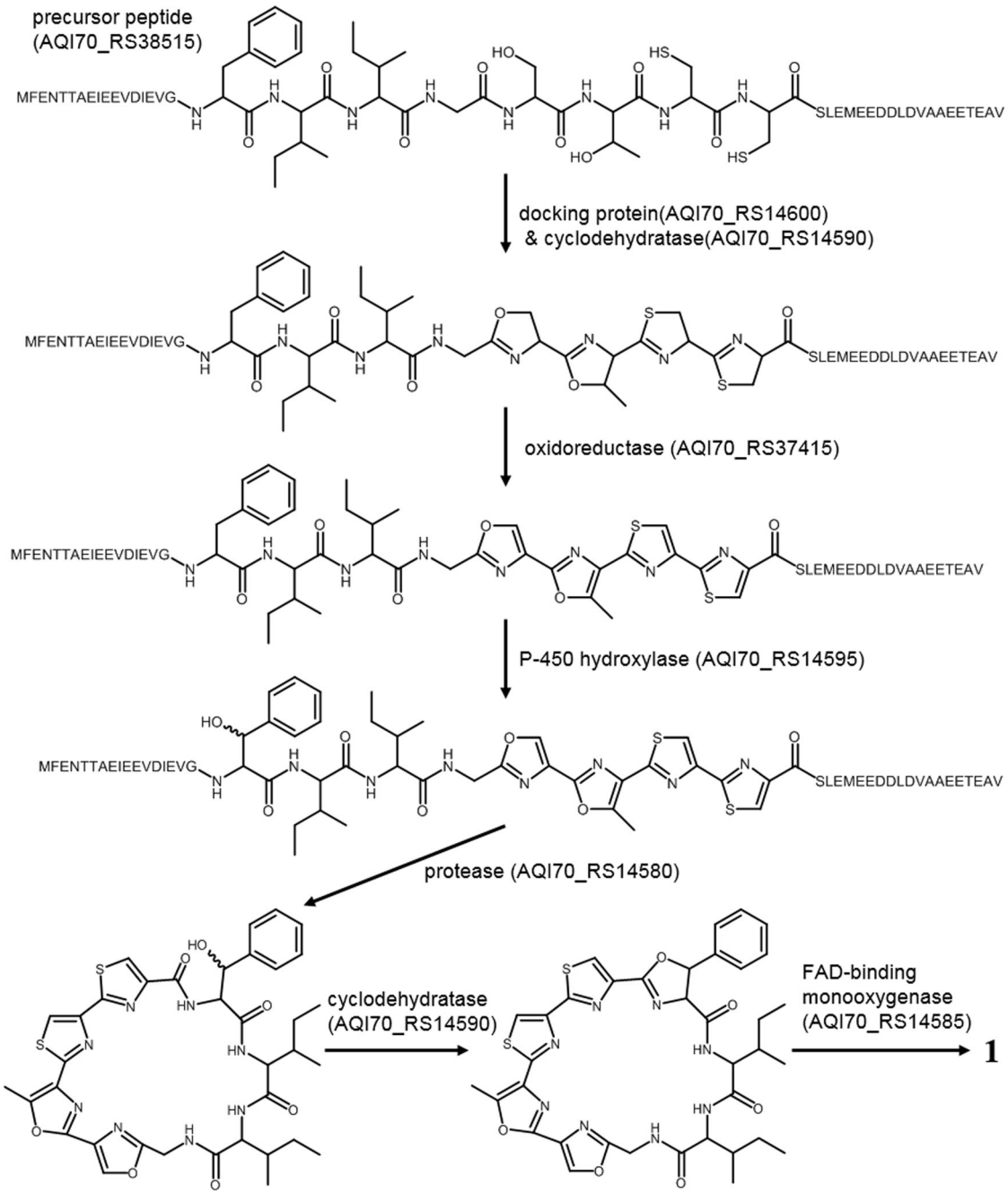

Fig. 5 Biosynthetic pathway of curacozole (**1**)

3 min. The reaction mixture was cooled down at room temperature before it was neutralized with 50 µL of 2N HCl and diluted with 1 mL of 50% MeCN. For the standard amino acids, each amino acid was derivatized with L-FDLA and D-FDLA using the same method. Approximately 30 µL of each FDLA derivative was subjected to HPLC analysis on a C18 column (4.6 × 250 mm, Wakopak Handy ODS, Wako). A DAD (MD-2018, JASCO, Tokyo, Japan) was used for the detection of the amino acid derivatives using the absorbance data from 220 nm to 420 nm. The HPLC analysis was performed at a flow rate of 1 mL/min using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) with a linear gradient program from 0 min to 70 min and increasing the percentage of solvent B from 25% to 60%. The retention times (min) of L- and D-FDLA derivatized amino acids in this HPLC condition were the following; L-allo-Ile-L-FDLA (41.20 min), L-Ile-L-FDLA (42.22 min), L-Ile-D-FDLA (58.16 min) and L-allo-Ile-D-FDLA (58.16 min).

Chiral HPLC Analysis

The hydrolysate of the peptide was analyzed by chiral HPLC on a SUMICHIRAL OA5000 (150 × 4.6 mm, Sumika Chemical Analyervice). The HPLC analysis was performed with UV detection at 254 nm using 2 mM $CuSO_4$ containing 5% MeCN as a mobile phase and a flow rate of 1 mL/min. The retention time of the amino acids in this HPLC condition were the following; D-allo-Ile (12.72 min) and D-Ile (15.90 min).

Cytotoxic assay

HCT116 and HOS cells (2.5×10^3) were aliquoted in 96-well plates and treated with **1** (5–100 nM) in D-MEM (HCT116) or E-MEM (HOS) containing FBS (10%). Cell viability was assayed after 72 h by using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, USA) with a JNR Luminescencer (ATTO, Tokyo, Japan) according to the manufacturer's protocol.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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