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Solid-Phase Total Synthesis of Cherimolacyclopeptide E and Discovery of More Potent Analogues by Alanine Screening

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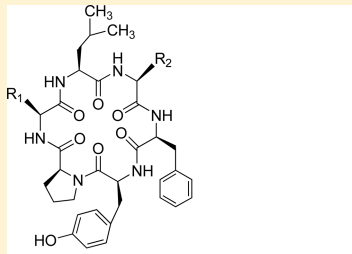
Supporting Information

ABSTRACT: Cherimolacyclopeptide E (**1**) is a cyclic hexapeptide obtained from *Annona cherimola*, reported to be cytotoxic against the KB (human nasopharyngeal carcinoma) cell line. The solid-phase total syntheses of this cyclic peptide and its analogues were accomplished by employing FMOC/*tert*-butyl-protected amino acids and the Kenner sulfonamide safety-catch linker. The synthetic peptide **1** was found to be weakly cytotoxic against four cell lines (MOLT-4, Jurkat T lymphoma, MDA-MB-231, and KB). Analogues **3** and **7**, where glycine at positions 2 and 6 of the parent compound was replaced by Ala, exhibited enhanced cytotoxicity against KB (**3**, IC_{50} 6.3 μM; **7**, IC_{50} 7.8 μM) and MDA-MB-231 breast cancer cells (**3**, IC_{50} 10.2 μM; **7**, IC_{50} 7.7 μM), thereby suggesting possible selective targeting of these cancer cells by these peptides. The spectral data of synthetic peptide **1** was found to be similar to that reported for the natural product. However, a striking difference in biological activity was noted, which warrants the re-evaluation of the original natural product for purity and the existence of conformational differences.



		KB cell line (IC_{50} μM)
1 (natural)	$R_1 = R_2 = H$	0.017
1 (synthetic)	$R_1 = R_2 = H$	>10
3	$R_1 = CH_3$, $R_2 = H$	6.3
7	$R_1 = H$, $R_2 = CH_3$	7.8

The fast-growing evergreen tree *Annona cherimola* Miller (Annonaceae) is native to the Andean mountain range in South America and is cultivated widely for its edible fruits. The plant is used locally in traditional medicines, and extracts have been shown to have antimicrobial activity.[1] A series of cytotoxic cherimolacyclopeptides have been isolated from extracts of the seeds of *A. cherimola* by Wélé et al.[2−7] Among these compounds, cherimolacyclopeptide E (**1**) was reported to exhibit cytotoxic activity against the KB (human nasopharyngeal carcinoma) cell line with an IC_{50} value of 0.017 μM.[6] Rajiv et al.[8] reported the solution-phase synthesis of cherimolacyclopeptide E (**1**) and its cytotoxicity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with CTC_{50} values of 2.76 and 4.96 μM, respectively, and antimicrobial activity against the pathogenic microbes *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*, with MICs between 6 and 12.5 μg/mL. Furthermore, this compound possesses a moderate anthelmintic activity against earthworms.[8] The biological activities reported for **1** have made it an attractive target for total synthesis and SAR studies. During the current study, a solid-phase approach, suitable for generating the natural product as well as its analogues by amino acid substitution was employed. For the synthesis, the Ellman variant of Kenner's sulfonamide "safety-catch" linker was used.[9,10] This strategy permits resin attachment through the backbone carboxylic acid of any residue and release of the cyclic peptide by head-to-tail cyclization on a solid phase. We have successfully used this methodology previously for the total syntheses of kahalalide A[11] and phakellistatin 12.[12]

RESULTS AND DISCUSSION

The total synthesis of cherimolacyclopeptide E (**1**) was achieved by solid-phase chemistry, using FMOC/*t*-Bu-protected amino acids and 4-sulfamylbutyryl resin as the solid support (Scheme 1). The starting FMOC-protected amino acid was loaded on 4-sulfamylbutyryl AM resin using benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling agent with *N*,*N*-diisopropylethylamine (DIEA). Leucine was chosen as the site of resin attachment, as it can undergo cyclization with glycine, and both are unhindered amino acids. The resulting loading level was determined by UV spectrophotometric analysis and found to be 0.517 mmol/g. The linear peptide was then synthesized by following standard FMOC protocols in which all the FMOC-protected amino acids were activated by hydroxybenzotriazole/diisopropylcarbodiimide (HOBt/DIC) in *N*,*N*-dimethylformamide (DMF). FMOC deprotection before each coupling step was achieved by the treatment of the resin-bound peptide with

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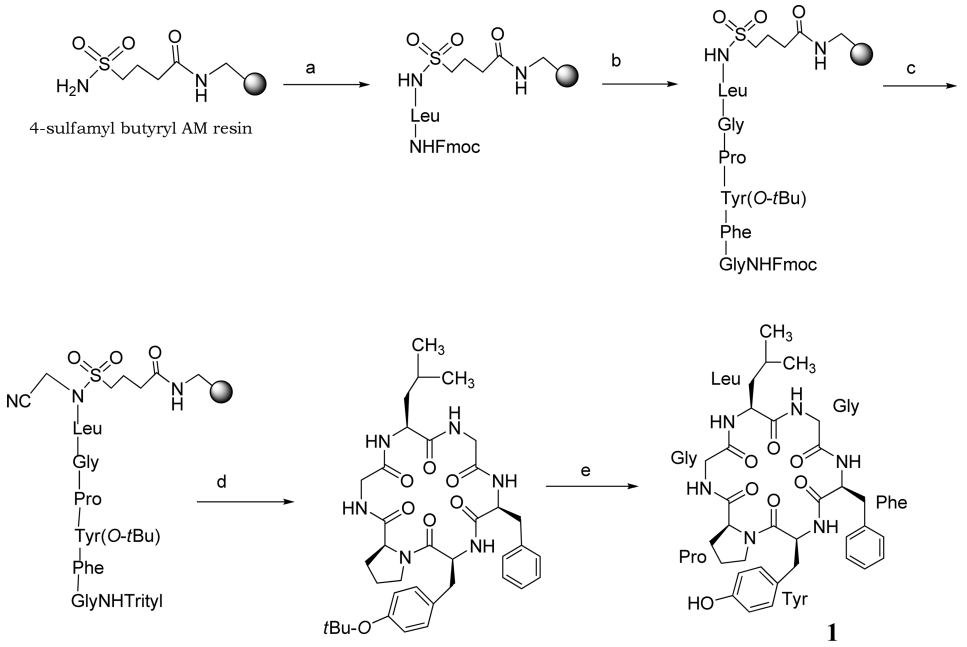
Scheme 1. Solid-Phase Synthesis of Cherimolacyclopeptide E (1)[a]



[a]Reagents and reaction conditions: PyBOP method (a) resin (1.1 mmol/g), FMOC-Leu (4 equiv), DIEA (8 equiv), PyBOP (4 equiv); (b) (i) 20% piperidine/DMF; (ii) FMOC-L-Gly-OH, DIC, HOBt (3 equiv each); (iii) 20% piperidine/DMF; (iv) FMOC-L-Pro-OH, DIC, HOBt (3 equiv each); (v) 20% piperidine/DMF; (vi) FMOC-L-Tyr (O*t*-Bu)-OH, DIC, HOBt (3 equiv each); (vi) 20% piperidine/DMF; (vii) FMOC-L-Phe-OH, DIC, HOBt (3 equiv each); (viii) 20% piperidine/DMF, (ix) FMOC-L-Gly-OH, DIC, HOBt (3 equiv each); (c) (i) 20% piperidine/DMF (ii) Trt-Cl (4 equiv), DIEA (8 equiv); (iii) ICH_2CN (10 equiv), DIEA (12 equiv); (d) 5% TFA/CH_2Cl_2, DIEA/THF; (e) TFA/CH_2Cl_2/TIS (1:1:0.01).

a 20% solution of piperidine in DMF, and the resin was subjected to coupling−deprotection steps to build the linear hexapeptide as the precursor for the cyclic peptide **1** (Scheme 1). After the construction of the linear peptide, the *N*-terminal FMOC protecting group was removed and replaced by the trityl group, followed by sulfonamide alkylation with iodoacetonitrile, which activated the safety-catch linker. The trityl deprotection to the free amine and subsequent treatment with DIEA in THF resulted in macrocyclization and release of the cyclic peptide **1** (Scheme 1) with the *tert*-butyl protecting group still intact. Solution-phase treatment with TFA/CH_2Cl_2/TIS (triisopropylsilane) yielded the crude peptide **1**.

The crude peptide was concentrated, followed by precipitation in diethyl ether. The identity of the product was validated by ESIMS, and its purity was checked by HPLC. Crude peptide **1** was dissolved in CH_3CN/H_2O (1:2) and purified by recycling preparative HPLC to obtain **1** in an overall yield of 9.8%.

The structure of **1** was deduced by mass spectrometry and 1D- and 2D-NMR spectroscopy. The molecular mass of **1** was determined by HRESIMS, as *m*/*z* 635.3157 $[M + H]^+$. The ^1H NMR data were recorded in d_5-pyridine, as this was the solvent used for the NMR studies of the naturally isolated cherimolacyclopeptide E (**1**). The ^1H NMR data of **1** showed the five amide protons at δ 11.24 (2H, s, Gly-*NH*CO), 9.98 (1H, brs, Phe_5-*NH*CO), 9.89 (1H, brs, Leu_1-*NH*CO), and 8.80 (1H, s, Tyr_4-*NH*CO), whereas the alpha protons were observed at δ 5.64 (1H, brs, Phe_5-*CH*), 5.64 (1H, m, Tyr_4-*CH*), 5.26 (1H, m, Leu_1-CH), and 4.47 (1H, brs, Pro_3-CH) (Table S1, Supporting Information).

^{13}C NMR spectroscopy provides useful information about *cis*−*trans* isomerism in proline-containing peptides. ^{13}C NMR chemical shifts for Pro in *cis* and *trans* configurations vary significantly, both in small peptides and in proteins.[13] The ^{13}C NMR data of **1** indicated the presence of a *trans*-tyrosyl-proline amide bond, as shown by the small ^{13}C NMR chemical shift difference of Pro, $\Delta\delta_{C_\beta-C_\gamma}$ = 4.6 ppm.[13−15] The same value was reported for naturally isolated **1**, indicating that the natural and synthetic samples both have the *trans*-tyrosyl-proline amide bond. However, slight broadening of signals was observed for the alpha protons of the leucine and proline residues of **1**, indicating the possibility that some conformational equilibrium exists for this compound. The overall NMR data for synthetic peptide **1** showed a close resemblance with reported data of the natural product (Table S1, Supporting Information). Moreover, the NMR data differed from that reported for cherimolacyclopeptide E, as synthesized by Rajiv et al., and we attribute this to use of different solvents (i.e., $CDCl_3$ rather than pyridine-d_6). For example, Rajiv et al. reported the alpha protons for Leu in the range δ 6.26−6.22 in $CDCl_3$, whereas in pyridine, a chemical shift of 5.26 ppm was observed.

With the solid-phase synthesis of **1** successfully completed, several alanine-substituted analogues of **1** were synthesized. The constituent amino acids of **1** were sequentially substituted by alanine, one at a time through the "alanine scanning" technique.[16] All of the amino acids of the natural product were found to be of the L-series, and therefore L-alanine was used for the replacement of different amino acids in the parent molecule.

The alanine-substituted linear precursors were synthesized and cyclized to produce analogues **2**−**7**. In addition, analogues **8** and **9** were prepared in which Pro was replaced by the more polar acidic amino acid glutamic acid and the less hindered glycine, respectively, to observe the effect of proline substitution by these amino acids on the resultant cytotoxicity. All analogues were subjected to product identification by

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Table 1. Cherimolacyolpeptide E (1) and Alanine-Substituted Analogues 2−9

compound	substituted residue	m/z $[M + H]^+$ (mol. formula, calcd)	sequence of compound
1	none	635.3157 ($C_{33}H_{43}N_6O_7$, 635.3193)	cyclo (Leu-Gly-Pro-Tyr-Phe-Gly)
2	Leu	593.2776 ($C_{30}H_{37}N_6O_7$, 593.2724)	cyclo (Ala-Gly-Pro-Tyr-Phe-Gly)
3	Gly	649.3379 ($C_{34}H_{45}N_6O_7$, 649.3350)	cyclo (Leu-Ala-Pro-Tyr-Phe-Gly)
4	Pro	609.3102 ($C_{31}H_{41}N_6O_7$, 609.3037)	cyclo (Leu-Gly-Ala-Tyr-Phe-Gly)
5	Tyr	543.2981 ($C_{27}H_{39}N_6O_6$, 543.2931)	cyclo (Leu-Gly-Pro-Ala-Phe-Gly)
6	Phe	559.2815 ($C_{27}H_{39}N_6O_7$, 559.2880)	cyclo (Leu-Gly-Pro-Tyr-Ala-Gly)
7	Gly	649.3311 ($C_{34}H_{45}N_6O_7$, 649.3350)	cyclo (Leu-Gly-Pro-Tyr-Phe-Ala)
8	Pro	667.3119 ($C_{34}H_{45}N_6O_7$, 667.3086)	cyclo (Leu-Gly-Glu-Tyr-Phe-Gly)
9	Pro	595.2819 ($C_{30}H_{39}N_6O_7$, 595.2874)	cyclo (Leu-Gly-Gly-Tyr-Phe-Gly)

ESIQTOFMS and MS/MS analysis. Results are summarized in Tables 1 and 2.

The cytotoxic effects of cherimolacyclopeptide E (**1**) and its analogues on the KB, MOLT-4, Jurkat T lymphoma, and MDA-MB-231 cancer cell lines were evaluated by employing a cell viability assay (Figure S3, Supporting Information).

Cherimolacyclopeptide E (**1**), as isolated from the natural source, was reported to have potent cytotoxic activity against the KB (human nasopharyngeal carcinoma) cell line, with an IC_{50} value of 0.017 μM.[5] However, **1** when synthesized in our laboratory was found to elicit no cytotoxic effect (IC_{50} >10 μM) against the KB, MOLT-4, Jurkat T, and MDA-MB231 cancer cell lines (Table 3).

Even though the synthetic peptide **1** has the same *trans*-tyrosyl-proline amide bond as present in the natural product, it did not show the same in vitro cytotoxicity against various cancer cell lines including the KB cell line. Several investigators have reported that in some cases synthetic proline-containing cyclic peptides are chemically equivalent to their natural counterparts, while deviating in their biological activities.[17−20] This has been attributed to conformational differences or the presence of biologically active impurities in the naturally isolated material. The same reasons may explain the variation in biological activity of synthetic and natural cherimolacyclopeptide E (**1**). This also suggests the need for a careful re-evaluation of the highly purified natural product against the KB cell line under identical experimental conditions.

The screening of derivatives of **1** against the KB cell line provided interesting results. Analogues **3** and **7**, where the Gly residues were replaced with alanine, displayed a significant antiproliferative activity against the MDA-MB231 and KB cell lines (Table 3). As both of these compounds are Gly replacements, and Gly is the most flexible amino acid, this further suggests that cherimolacyclopeptide E is susceptible to conformational equilibration, which is minimized by Ala substitution in **3** and **7**.

EXPERIMENTAL SECTION

General Experimental Procedures. All FMOC amino acids, coupling reagents, and resins were purchased from Novabiochem, and other chemicals were from Aldrich. Dichloromethane and triethylamine were distilled over CaH_2, and THF was distilled over Na in the presence of benzophenone. Optical rotations were measured on a JASCO DIP 360 polarimeter. UV spectra were recorded on a Hitachi 3200 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on Bruker NMR spectrometers, operating at 600 MHz (125 MHz for ^{13}C). HRFABMS was recorded on JEOL JMS HX 110 mass spectrometers. Chemical shifts are reported in ppm. A LC-908W recycling preparative HPLC with a Jaigel-polyamine PBMIN-5-A column and an analytical HPLC Waters 996 with photodiode array detector was used.

Peptide Synthesis. To the 4-sulfamylbutyryl AM resin (Novabiochem, 1.1 mmol/g) preswollen in DMF were added FMOC-amino acid (4 equiv), PyBOP (2.28 g, 4 equiv), and DIEA (1.5 mL, 8 equiv) in 5 mL of DMF. The reaction mixture was left overnight, and the coupling repeated twice more. The efficiency of loading was determined by FMOC removal analysis.

Peptide Coupling. The FMOC-peptidyl resin, swollen in DMF, was deprotected by 20% piperidine in DMF for 2 min, and the deprotection repeated for 20 min. The resin was washed (3 × DMF) and the presence of the amino group was checked with the Kaiser test. At the same time, the next FMOC amino acid (3 equiv) was activated in 5 mL of DMF by DIC (3 equiv, 514 μL) and HOBt (3 equiv, 505.2 mg). The solution was filtered in case of precipitation before addition to the deprotected peptidyl resin. Completion of reaction was checked by a ninhydrin colorimetric test. Elongation of the linear peptide was continued, until the FMOC group on the last residue was removed.

"Safety-Catch" Activation and Cyclative Cleavage of Cyclic Peptides. The resin was treated with trityl chloride (1.227 g, 4 equiv) and DIEA (1.53 mL, 8 equiv) for 2 h. After DMF washing, this step was repeated under the same conditions. The sulfonamide linker was activated by iodoacetonitrile (0.8 mL, 10 equiv) and DIEA (2.3 mL, 12 equiv) in 5.0 mL of *N*-methylpyrrolidinone for 12 h. This activation step was repeated under the same conditions. After resin washing (*N*-methylpyrrolidinone, CH_2Cl_2), the trityl group was removed by 5% TFA in CH_2Cl_2 treatment for 2 min. The deprotection was repeated for 2 h, followed by a Kaiser test. The linear peptidyl resin was then washed (3 × CH_2Cl_2, 3 × THF, once by 1% DIEA in THF). Immediately afterward, the resin was swollen in THF, and DIEA (565.5 μL, 3 equiv) added. The cyclization proceeded overnight, releasing the cyclic peptide **1** with a *tert*-butyl group, which was deprotected by TFA/CH_2Cl_2/TIS (1:1:0.01).

Mass Spectrometric Analysis of Cherimolacyclopeptide E (1) and Its Analogues. Matrix-assisted laser desorption/ionization (MALDI) was carried out on an Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer. All cyclic peptides (5 pmol in MeOH/H_2O, 1:1, with 0.1% TFA) were mixed with 0.5 μL of the matrix (DHB) solution (saturated solution in 0.1% TFA/CH_3CN, 2:1) and deposited on a MALDI plate. Mass was recorded with the ion source 1 (IS1) set to 25.00 kV and ion source 2 (IS2) set to 21.50 kV and without delay extraction (DE). These experiments were performed with the laser energies of 70−80%. The LIFT experiment (MS/MS) was performed on the same instrument with IS1 set to 8.00 kV and IS2 set to 7.15 kV, and LIFT 1 set to 19.00 kV and without DE. The laser energies used were 75−80%. The validation of data obtained, including baseline subtraction of the TOF data, external calibration using peptide calibration standard (Bruker Daltonics, Bremen, Germany), and all further data processing was carried out by using Flex analysis 2.0 postanalysis software and for data acquisition by Flex control 2.0.

ESIQTOFMS spectra were recorded on a Q-STAR XL mass spectrometer (Applied Biosystems). Each cyclic peptide (5 pmol in MeOH/H_2O, 1:1, with 0.1% TFA) was infused directly into the mass spectrometer at a flow rate of 3 μL/min to acquire full scan and product ion mass spectra. The electrospray voltage at the spraying needle was optimized at 5200 V. Low-energy collision-induced dissociation (CID) experiments were performed using nitrogen

Table 2. Mass Spectrometric Analysis of 1 and Its Analogues

compound	structure	$[M + H]^+$ (mol,. formula, calcd)	MS/MS frag. of $[M + H]^+$	proposed fragment structure
1	cyclo(Leu^1-Gly^2-Pro^3-Tyr^4-Phe^5-Gly^6)	635.3157 ($C_{33}H_{43}N_6O_7$, 635.3193)	607[a,b]	$[M - CO]^+$
			522[a]	$[H\text{-}Gly^2\text{-}Pro^3\text{-}Tyr^4\text{-}Phe^5\text{-}Gly^6]^+$
			472[a,b]	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6\text{-}Phe^5]^+$
			444[a,b]	$[(H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6\text{-}Phe^5) - CO]^+$
			368[a]	$[H\text{-}Tyr^4\text{-}Phe^5\text{-}Gly^6]^+$
			325[a,b]	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6]^+$
			297[b]	$[(H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6) - CO]^+$
			268[a,b]	$[H\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3]^+$
			240[a,b]	$[(H\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3) - CO]^+$
			175[a]	$[H\text{-}Phe^5\text{-}Gly^6\text{-}\ CO]^+$
			155[a,b]	$[H\text{-}Gly^2\text{-}Pro^3]^+$
			136[a]	immonium ion tyrosine
			70[a]	immonium ion proline
2	cyclo(Ala^1-Gly^2-Pro^3-Tyr^4-Phe^5-Gly^6)	593.2776 ($C_{30}H_{37}N_6O_7$, 593.2724)	565	$[M - CO]^+$
			430	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Ala^1\text{-}Gly^6\text{-}Phe^5]^+$
			402	$[(H\text{-}Pro^3\text{-}Gly^2\text{-}Ala^1\text{-}Gly^6\text{-}Phe^5)\text{-}CO]^+$
			368	$[H\text{-}Tyr^4\text{-}Phe^5\text{-}Gly^6]^+$
			333	$[H\text{-}Gly^2\text{-}Ala^1\text{-}Gly^6\text{-}Phe^5]^+$
			283	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Ala^1\text{-}Gly^6]^+$
			226	$[H\text{-}Ala^1\text{-}Gly^2\text{-}Pro^3]^+$
3	cyclo(Leu^1-Ala^2-Pro^3-Tyr^4-Phe^5-Gly^6)	649.3379 ($C_{34}H_{45}N_6O_7$, 649.3350)	592	$[H\text{-}Leu^1\text{-}Ala^2\text{-}Pro^3\text{-}Tyr^4\text{-}Phe^5]^+$
			558	$[H\text{-}Gly^6\text{-}Leu^1\text{-}Ala^2\text{-}Pro^3\text{-}Tyr^4]^+$
			486	$[H\text{-}Phe^5\text{-}Gly^6\text{-}Leu^1\text{-}Ala^2\text{-}Pro^3]^+$
			339	$[H\text{-}Gly^6\text{-}Leu^1\text{-}Ala^2\text{-}Pro^3]^+$
			136	immonium ion tyrosine
			70	immonium ion proline
4	cyclo(Leu^1-Gly^2-Ala^3-Tyr^4-Phe^5-Gly^6)	609.3102 ($C_{31}H_{41}N_6O_7$, 609.3037)	581	$[M - CO]^+$
			552	$[H\text{ -}Leu^1\text{-}Gly^6\text{-}Phe^5\text{-}Tyr^4\text{-}Ala^3]^+$
			495	$[H\text{-}Gly^6\text{-}Phe^5\text{-}Tyr^4\text{-}Ala^3\text{-}Gly^2]^+$
			446	$[H\text{- }Ala^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6\text{-}Phe^5]^+$
			375	$[H\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6\text{-}Phe^5]^+$
			299	$[H\text{-}Ala^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6]^+$
			136	immonium ion tyrosine
5	cyclo(Leu^1-Gly^2-Pro^3-Ala^4-Phe^5-Gly^6)	543.2981 ($C_{27}H_{39}N_6O_6$, 543.2931)	515	$[M - CO]^+$
			472	$[H\text{-}Phe^5\text{-}Gly^6\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3\ ]^+$
			430	$[H\text{-}Gly^2\text{-}Pro^3\text{-}Ala^4\text{-}Phe^5\text{-}Gly^6]^+$
			396	$[H\text{-}Gly^6\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3\text{-}Ala^4]^+$
			325	$[H\text{- }Gly^6\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3]^+$
			268	$[H\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3]^+$
			226	$[H\text{-}Gly^2\text{-}Pro^3\text{-}Ala^4]^+$
			171	$[H\text{-}Leu^1\text{-}Gly^2]^+$
			155	$[H\text{-}Gly^2\text{-}Pro^3]^+$
			136	immonium ion tyrosine
			70	immonium ion proline
6	cyclo(Leu^1-Gly^2-Pro^3-Tyr^4-Ala^5-Gly^6)	559.2815 ($C_{27}H_{39}N_6O_7$, 559.2880)	531	$[M - CO]^+$
			502	$[H\text{-}Pro^3\text{-}Tyr^4\text{-}Ala^5\text{-}Gly^6\text{-}Leu^1]^+/[H\text{-}Ala^5\text{-}Tyr^4\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1]^+$
			445	$[H\text{-}Gly^2\text{-}Pro^3\text{-}Tyr^4\text{-}Ala^5\text{-}Gly^6]^+$
			396	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6\text{-}Ala^5\ ]^+$
			325	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Gly^6]^+$
			268	$[H\text{-}Leu^1\text{-}Gly^2\text{-}Pro^3]^+$
			155	$[H\text{-}Gly^2\text{-}Pro^3]^+$
7	cyclo(Leu^1-Gly^2-Pro^3-Tyr^4- Phe^5-Ala^6)	649.3311 ($C_{34}H_{45}N_6O_7$, 649.3350)	621	$[M - CO]^+$
			592	$[H\text{-}Leu^1\text{-}Ala^6\text{-}Phe^5\text{-}Tyr^4\text{-}Pro^3]^+$
			486	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Ala^6\text{-}Phe^5]^+$
			339	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1\text{-}Ala^6\ ]^+$
			268	$[H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1]^+$
			240	$[(H\text{-}Pro^3\text{-}Gly^2\text{-}Leu^1)- CO]^+$
			155	$[H\text{-}Gly^2\text{-}Pro^3]^+$
			136	immonium ion tyrosine

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Table 2. continued

compound	structure	$[M + H]^+$ (mol,. formula, calcd)	MS/MS frag. of $[M + H]^+$	proposed fragment structure
			70	immonium ion proline
8	Leu[1]-Gly[2]-Glu[3]-*Tyr[4]-Phe[5]-Gly[6]	667.3119 ($C_{33}H_{43}N_6O_9$, 667.3086)	649	$[M + H - H_2O]^+$
			639	$[M + H - CO]^+$
			610	[H-Phe[5]-Tyr[4]-Glu[3]-Gly[2]-Leu[1]]$^+$
			554	[H-Gly[6]-Phe[5]-Tyr[4]-Glu[3]-Gly[2]]$^+$
			504	[H-Glu[3]-Gly[2]-Leu[1]-Gly[6]-Phe[5]]$^+$
			486	[H-Glu[3]-Gly[2]-Leu[1]-Gly[6]-Phe[5] $- H_2O$]$^+$
			458	[H-Glu[3]-Gly[2]-Leu[1]-Gly[6]-Phe[5] $- H_2O - CO$]$^+$
			339	[H-Glu[3]-Gly[2]-Leu[1]-Gly[6] $- H_2O$]$^+$
			282	[H-Glu[3]-Gly[2]-Leu[1] $- H_2O$]$^+$
			136	immonium ion tyrosine
9	Leu[1]-Gly[2]-Gly[3]-Tyr[4]-Phe[5]-Gly[6]	595.2819 ($C_{30}H_{39}N_6O_7$, 595.2874)	577	$[M + H - H_2O]^+$
			567	$[M + H - CO]^+$
			482	[H-Gly[6]-Phe[5]-Tyr[4]-Gly[3]-Gly[2]]$^+$
			448	[H-Tyr[4]-Gly[3]-Gly[2]-Leu[1]-Gly[6]]$^+$
			425	[H-Gly[6]-Phe[5]-Tyr[4]-Gly[3]]$^+$
			391	[H-Tyr[4]-Gly[3]-Gly[2]-Leu[1]]$^+$
			368	[H-Gly[6]-Phe[5]-Tyr[4]]$^+$
			285	[H-Gly[3]-Gly[2]-Leu[1]-Gly[6]]$^+$
			278	[H-Tyr[4]-Gly[3]-Gly[2]]$^+$
			221	[H-Tyr[4]-Gly[3]]$^+$
			205	[H-Gly[6]-Phe[5]]$^+$
			136	immonium ion tyrosine

[a]Obtained from MALDI-TOF-TOF-MS. [b]Obtained from ESIQTOFMS.

Table 3. IC_{50} Values of Peptide 1 and Alanine-Substituted Analogues 2−9

compound	cell line (IC_{50} μM) KB	MDA-MB-231
1	>10	>10
2	>10	>10
3	6.3	10.2
4	>10	>10
5	>10	>10
6	>10	>10
7	7.8	7.7
8	>10	>10
9	>10	>10
doxorubicin (positive control)	0.4	0.04

(CID gas valve set to 4) as collision gas, and a collision energy of 35 eV was used.

*cyclo-Leu[1]-Gly[2]-Pro[3]-Tyr (OH)[4]-Phe[5]-Gly[6] (Synthetic Cherimola-cyclopeptide E, **1**).* Yield 9.8%; $[\alpha]^{23}_D$ −46.0 (*c* 0.3, MeOH, lit. $[\alpha]^{23}_D$ −56.0 (*c* 0.005, MeOH); UV (MeOH) λ_{max} 267, 254, 226, nm; IR (KBr) ν_{max} 3310, 2922, 1693, 1562, 1514, 1413 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table S1; HRMS (ESI+) *m*/*z* 635.3157 $[M + H]^+$ ($C_{33}H_{43}N_6O_7$, calcd for 635.3193).

*cyclo-Ala[1]-Gly[2]-Pro[3]-Tyr(OH)[4]-Phe[5]-Gly[6] (**2**).* The protected cyclic peptide **2a** (4.5 mg) was dissolved in a mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **2** as an amorphous solid. Yield 1.4%; $[\alpha]^{23}_D$ −10.8 (*c* 0.06, MeOH); UV(MeOH) λ_{max} 295, 286, 275, 269, 225, nm; IR (KBr) ν_{max} 3292, 2923, 2858, 1678, 1560, 1452 cm^{-1}; FABMS *m*/*z* 593 $[M + H]^+$; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Ala[2]-Pro[3]-Tyr (OH)[4]-Phe[5]-Gly[6] (**3**).* The protected cyclic peptide **3a** (5.0 mg) was dissolved in a mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo to obtain the crude cyclic peptide, which was then purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **3** as an amorphous solid. Yield 0.8%; $[\alpha]^{23}_D$ −20.3 (*c* 0.03, MeOH); UV(MeOH) λ_{max} 285, 270, 261, 223, nm; IR (KBr) ν_{max} 3255, 2922, 2856, 1681, 1562, 1417 cm^{-1}; FABMS *m*/*z* 649 $[M + H]^+$; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Gly[2]-Ala[3]-Tyr(OH)[4]-Phe[5]-Gly[6] (**4**).* The protected cyclic peptide **4a** (5 mg) was dissolved in a mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **4** as an amorphous solid. Yield 4.6%; $[\alpha]^{23}_D$ −4.8 (*c* 0.1, MeOH); UV(MeOH) λ_{max} 270, 265, 240, nm; IR (KBr) ν_{max} 3745, 2925, 2858, 1678, 1624, 1454 cm^{-1}; FABMS *m*/*z* 609 $[M + H]^+$; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Gly[2]-Pro[3]-Ala[4]-Phe[5]-Gly[6] (**5**).* Yield 4.9%; $[\alpha]^{23}_D$ −8.9 (*c* 0.06, MeOH); UV(MeOH) λ_{max} 288, 275, 253, 222, nm; IR (KBr) ν_{max} 3622, 2921, 2862, 1681, 1461 cm^{-1}; FABMS *m*/*z* 543 $[M + H]^+$; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Gly[2]-Pro[3]-Tyr (OH)[4]-Ala[5]-Gly[6] (**6**).* The protected cyclic peptide **6a** (5.5 mg) was dissolved in a mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **6** as a gummy material. Yield 2.2%; $[\alpha]^{23}_D$ −11.9 (*c* 0.04, MeOH); UV(MeOH) λ_{max} 275, 246, 223, nm; IR (KBr) ν_{max} 3722, 2925, 2862, 1732, 1683, 1458 cm^{-1}; FABMS *m*/*z* 559 $[M + H]^+$; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Gly[2]-Pro[3]-Tyr (OH)[4]-Phe[5]-Ala[6] (**7**).* The protected cyclic peptide **7a** (10 mg) was dissolved in a mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 2 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **7** as a gummy material. Yield 1.7%; $[\alpha]^{23}_D$ −5.1 (*c* 0.04, MeOH); UV(MeOH) λ_{max} 286, 252, 250, nm; IR (KBr) ν_{max} 3300, 2923, 2858, 1733, 1674, 1560, 1456 cm^{-1}; FABMS *m*/*z* 649; MS/MS (ESI+), see Table 2.

*cyclo-Leu[1]-Gly[2]-Glu[3]-Tyr (OH)[4]-Phe[5]-Gly[6] (**8**).* The protected cyclic peptide (4 mg) was dissolved in a 2 mL mixture of TFA/DCM/TIS

(1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **8** as a gummy material. Yield 4.2%; $[\alpha]^{23}_D$ −10.7 (*c* 0.05, MeOH); UV(MeOH) λ_{max} 299, 276, 264, 247, nm; IR (KBr) ν_{max} 2925, 2860, 1733, 1552, 1510, 1460 cm^{-1}; FABMS *m/z* 667 $[M + H]^+$; MS/MS (ESI+), see Table 2.

cyclo-Leu^1*-Gly*^{2}*-Gly*^{3}*-Tyr (OH)*^{4}*-Phe*^{5}*-Gly*^{6} (**9**). The protected cyclic peptide (4 mg) was dissolved in a 2 mL mixture of TFA/DCM/TIS (1:1:0.01) and reacted for 1 h at room temperature. The reaction mixture was concentrated in vacuo and purified on silica gel preparative TLC plates (5% MeOH/$CHCl_3$) to obtain compound **9** as a gummy material. Yield 3.1%; $[\alpha]^{23}_D$ −37.7 (*c* 0.01, MeOH); UV(MeOH) λ_{max} 298, 277, 265, 248 nm; IR (KBr) ν_{max} 3255, 2922, 2856, 1681, 1562, 1417 cm^{-1}; FABMS *m/z* 595 $[M + H]^+$; MS/MS (ESI+), see Table 2.

Cytotoxicity Assays. Human KB cells, human MDA-MB-231 breast cancer cells, MOLT-4, and Jurkat leukemia cells were seeded in a 96-well plate at the cell densities of 4×10^3 cells/well. After overnight incubation, the cells were treated with increasing concentrations (ranging between 0.064 and 40 μM) of parent cherimolacyclopeptide E (**1**) and its corresponding alanine-substituted analogues **2**−**9**. The compounds were first dissolved in DMSO and then diluted to the desired concentration with culture medium. After 72 h incubation, CellTiter 96 Aqueous Cell Proliferation Reagent (Promega, Madison, WI, USA), which is composed of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] and the electron coupling reagent PMS (phenazine methosulfate), was added to each well, according to the manufacturer's instructions. The cell viability was determined by measuring the absorbance at 490 nm by using a microplate ELISA reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Results were shown as the average cell viability $[(OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%]$ of triplicate wells. Untreated cells (DMSO + medium) served as the positive control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete NMR data of synthetic and natural peptide **1** and cytotoxicity data of synthetic peptides **1**−**9**; ^1H spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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