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The Complestatins as HIV-1 Integrase Inhibitors. Efficient Isolation, Structure Elucidation, and Inhibitory Activities of Isocomplestatin, Chloropeptin I, New Complestatins, A and B, and Acid-Hydrolysis Products of Chloropeptin I

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Received December 29, 2000

From the screening of a microbial extract library, isocomplestatin (**1**), a new axial-chiral isomer of complestatin (**2**) which is a known rigid bicyclic hexapeptide, was identified as a potent natural product inhibitor of HIV-1 integrase, a unique enzyme responsible for viral replication. Isocomplestatin showed inhibitory activities (IC_{50}) in coupled 3′-end processing/strand transfer (200 nM), strand transfer (4 μM), and HIV-1 replication (200 nM) in virus-infected cells. Attempted large-scale isolation of **1** by the literature method, used for the isolation of complestatin, led to lower yield and limited availability. We have developed several new, two-step, high-yielding absorption/elution methods of isolation based on reverse-phase chromatography at pH 8 that are applicable to scales from one gram to potential industrial quantities. We have also discovered and determined the structure of two new congeners of **1**, namely, complestatins A (**4**) and B (**5**), with almost equal HIV-1 integrase activity. They differ from **1** at C2′ and C3′ of the tryptophan moiety (residue F). Selective acid hydrolysis of chloropeptin I (**3**), itself a known acid-catalyzed rearranged isomer of **1** and **2** (8′- vs 7′-substitution in tryptophan residue F, respectively), an isomer of complestatin, and isocomplestatin resulted in a number of fragments (**6**−**10**) with retention of most of the HIV-1 integrase activity. The structure−activity relationship as revealed by these compounds could possibly lead to the design of better inhibitors or understanding of the HIV-1 integrase target.

AIDS is caused by the human immunodeficiency virus (HIV) and remains one of the leading causes of loss of human lives worldwide. HIV-1 encodes three enzymes, (i) reverse transcriptase, (ii) integrase, and (iii) protease, that are collectively responsible for viral replication and infectivity. The discovery of many approved therapeutic agents that inhibit reverse transcriptase and protease has been helpful in prolonging and in some cases saving lives of AIDS-infected patients. Integrase is responsible for the multistep process of integration into the host cell genome that includes cleavage of two nucleotide bases from the 3′-end of each strand of viral DNA (3′-end processing) and transfer of the processed 3′-ends into the host cell (human) target DNA (strand transfer).[1] Significantly, integrase is not present in host cells and is specific to retroviruses. Integration is essential for retroviral replication; therefore inhibition of integrase should provide a means for inhibiting HIV-1. The demonstration that compounds[1e] that inhibit the integrase-catalyzed strand transfer reaction can effectively block viral replication validates this approach for the discovery and development of a new class of antiretroviral agents targeting HIV-1 integrase.

Screening of natural product extracts provides an opportunity for the discovery of structurally diverse enzyme inhibitors and receptor ligands that are usually not found by screening synthetic chemical collections or combinatorial libraries. We have recently reported the discovery of a number of novel natural product inhibitors of HIV-1 integrase that include equisetin, phomasetin from *Phoma* sp.,[2] and integric acid from *Xylaria* sp.[3] Continued screening with the recombinant integrase[4] enzyme using an in vitro assay led to the discovery and identification of isocomplestatin (**1**) from *Streptomyces* sp. as a potent inhibitor of HIV-1 integrase. Isocomplestatin inhibited the coupled 3′-processing/strand transfer assay with an IC_{50} of 0.2 μM. Isocomplestatin also inhibited the strand transfer activity of recombinant integrase in staged assays and integration using isolated HIV-1 preintegration complexes with comparable potency, IC_{50} values of 3−4 μM. Inhibition of HIV-1 replication was observed in cell culture with an IC_{50} of 0.2 μM.

Isocomplestatin (**1**), complestatin (**2**), and chloropeptin I (**3**) are bicyclic hexapeptides that differ at the position of the phenyl−indole ring junction. Complestatin (**2**) was originally isolated in 1980 as an inhibitor of an alternative pathway of complement (IC_{50} = 2 and 0.5 μM),[5a] and a planar structure was published nine years later.[5b−d] Chloropeptin I (**3**) was reported in 1994 along with complestatin (*a.k.a.* chloropeptin II) as inhibitors of gp120-CD4 binding (IC_{50} = 2−1.3 μM).[6] The absolute stereochemistry of chloropeptin I was determined in 1996 by a combination of hydrolysis, high-temperature molecular dynamics and Monte Carlo conformational searching, and NMR spectroscopy.[6d] More recently, chloropeptin I, which was prepared from HCl-catalyzed rearrangement of complestatin, was also shown to enhance the plasminogen binding and fibrinolysis.[7] Because of the structural complexities and various biological effects displayed by these compounds, they have attracted numerous efforts toward total synthesis,[8] but none have been successfully completed to date.

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10.1021/np000632z CCC: $20.00
Published on Web 06/15/2001

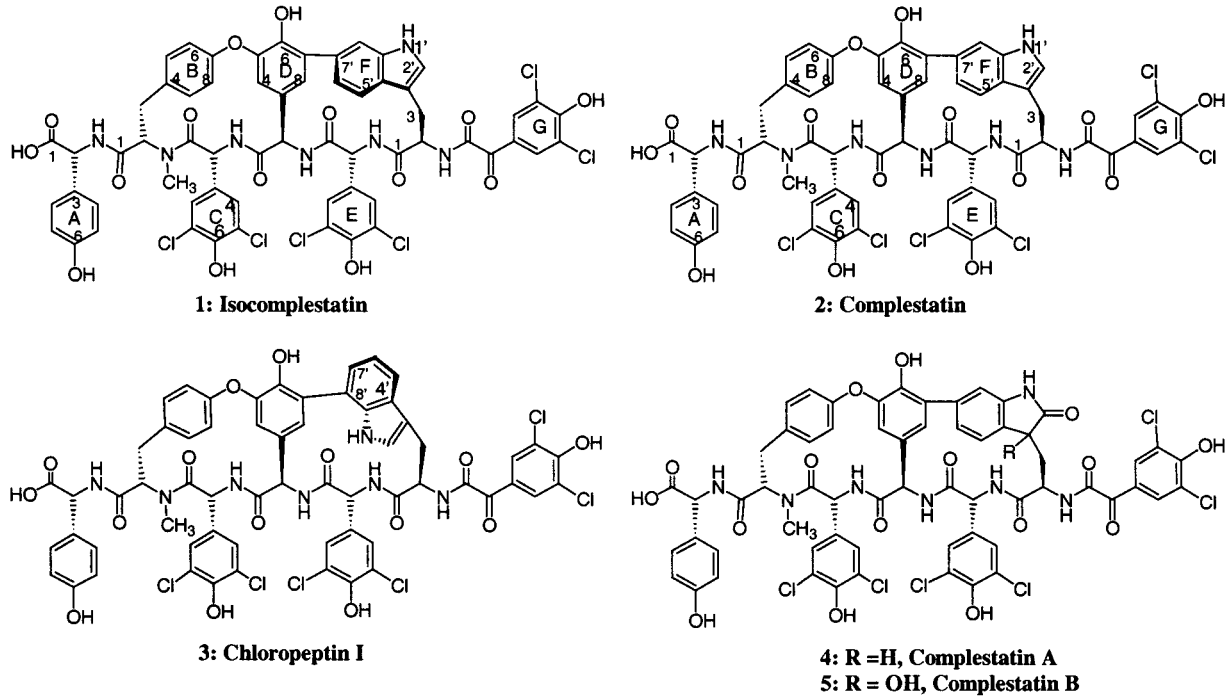

Figure 1. Structures of isocomplestatin (**1**), complestatin (**2**), chloropeptin I (**3**), and complestatins A (**4**) and B (**5**).

During the isolation of isocomplestatin (**1**) we observed that in the presence of mineral acid it was converted, at least in part, to chloropeptin I (**3**). We investigated this phenomenon and reported[9] an efficient TFA-catalyzed rearrangement of **1** to chloropeptin I (**3**). We also discovered two new congeners of isocomplestatin from cultured broth of *Streptomyces* sp., namely, complestatin A (**4**) and complestatin B (**5**), which possess oxidized tryptophan instead of tryptophan. Despite the structural similarities among these four compounds, their solubility behavior is highly dissimilar. The virtual insolubility of isocomplestatin in many common organic solvents presented a tremendous challenge for the large-scale isolation of this compound from fermentation broth. The inefficiencies and problems associated with existing methods for the isolation of complestatin and isocomplestatin prompted us to develop an efficient new method for its isolation to provide the needed quantities for chemical and biological studies. In this paper several methods for the isolation of isocomplestatin, stereochemical elucidation, the isolation and structure elucidation of the new complestatins A and B, the experimental details of the conversion of isocomplestatin to chloropeptin I, the detailed description of the hydrolysis[10] of chloropeptin I, and the anti HIV-1 activities of the natural products and derivatives are described.

Results and Discussion

Isolation of Isocomplestatin. (a) Bioassay-Guided Small-Scale Isolation of Isocomplestatin. Size exclusion chromatography (Sephadex LH-20) of methyl ethyl ketone extracts of the fermentation broth of *Streptomyces* sp. (MA7234) followed by reverse-phase HPLC gave isocomplestatin (50 mg/L).

(b) Larger Scale Isolation of Isocomplestatin. Attempts to scale-up the isolation of isocomplestatin by using the above method, the method described by Tanaka et al.[6b] for isolation of complestatin, and various other methods at lower pH (see Experimental Section) led to the conversion of isocomplestatin or complestatin[6b,9] to chloropeptin I due to low pH and resulted in an extremely poor recovery due to its poor solubility. We exhaustively examined various methods for the isolation of isocomplestatin that is free from chloropeptin I and developed a reverse-phase Amberchrom resin based absorption/elution method that is schematically described in Figure 2. In this method we took advantage of the anionic groups of isocomplestatin, which allowed the formation of salts with good solubility in aqueous methanol. Thus the fermentation broth filtrates and 70% aqueous acetone extracts of mycelia were separately chromatographed on Amberchrom resin, and the salts of isocomplestatin were eluted with 50% aqueous methanol. Treatment of the salts with aqueous citric acid in methanol followed by filtration and washing with solvents produced isocomplestatin in greater than 90% yield. This process readily led to the isolation of gram quantities of isocomplestatin and is potentially amenable for industrial scale isolation. Other resins such as HP20 and SP207 could be easily substituted

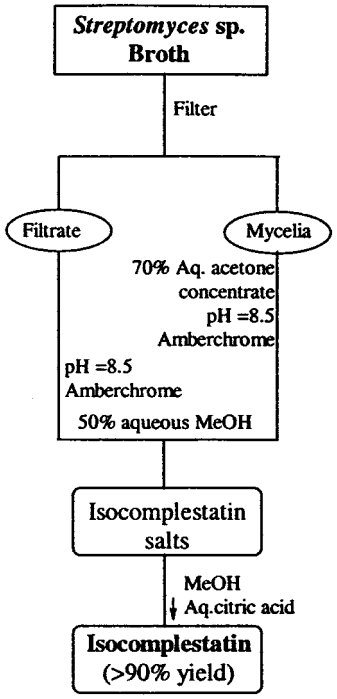

Figure 2. Isolation scheme of isocomplestatin.

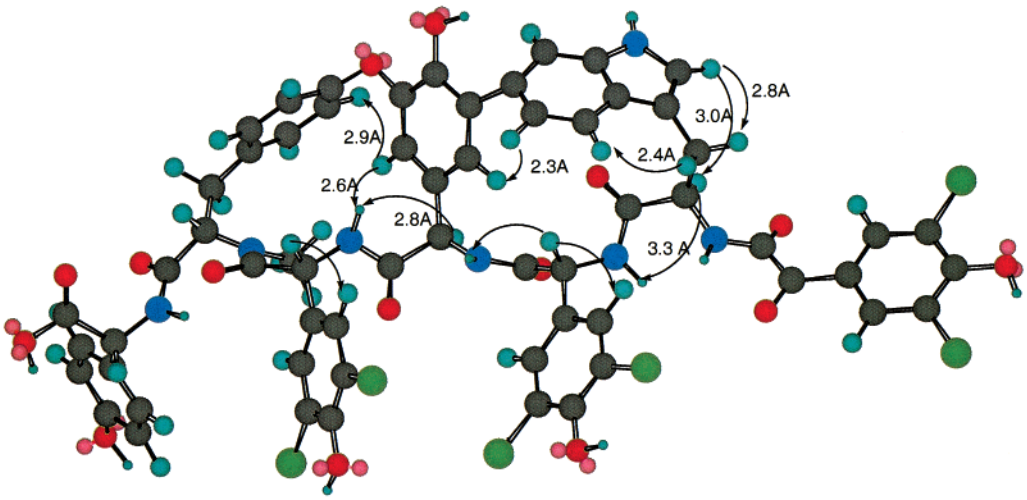

Figure 3. ChemDraw 3D model of isocomplestatin (**1**) showing NOESY correlations (mix = 300 ms).

for the isolation of isocomplestatin, but Amberchrom was found to be superior. Use of either mineral or the strong organic acids such as acetic or trifluoroacetic acids, during regeneration of free isocomplestatin from salts, caused significant formation of chloropeptin I.

Similar chromatography of the aqueous acetone extract or broth filtrate on Amberchrom resin at pH 2.5 and elution with 50−100% aqueous acetone at pH 2.5 gave excellent recovery of isocomplestatin. However, a significant portion (~15−40%) of isocomplestatin was converted to chloropeptin I. Therefore, these experiments further confirmed our previous observation that chloropeptin I is an isolation artifact. Since Tanaka et al.[6a] also used hydrochloric acid during their original isolation procedure of chloropeptin I, it is reasonable to assume that their chloropeptin I isolate is also an isolation artifact. However, Hegde et al.[11] recently reported the occurrence of chloropeptin I as a natural product.

Isolation of Complestatin A and B. Complestatins A and B together with a mixture of isocomplestatin and chloropeptin I were isolated from a 6 L fermentation of *Streptomyces* sp. MA7234. The fermentation broth was filtered, and the filtrate was acidified and extracted with ethyl acetate and chromatographed on Sephadex LH-20. Elution of the column with methanol followed by 1:1 methanol−acetone gave a mixture of four compounds. Purification of this mixture by reverse-phase HPLC gave the minor constituents complestatin B (**5**), complestatin A (**4**), and a mixture of isocomplestatin (**1**) and chloropeptin I (**3**). As shown earlier, there was a significant loss of isocomplestatin, during gel filtration on LH-20 and preparative HPLC, due to its insolubility, which helped in the relative enrichment and eventual isolation of minor congeners complestatins A and B. Like chloropeptin I, both complestatins A and B exhibited better solubility in methanol and other polar organic solvents. HPLC analysis of broth filtrates and the solvent washings of precipitated isocomplestatin from an Amberchrom column showed the presence of complestatins A and B, indicating that they are true natural products.

Structure Elucidation: Isocomplestatin (1) and Chloropeptin I (2). The physical, chemical, and spectral properties including ^{1}H and ^{13}C NMR spectra of **1** are identical to those of complestatin (**2**)[5,12] except for their specific rotations and the ^{1}H NMR chemical shifts of H-3 of tryptophan residue F, indicating that the two compounds were not completely identical. Compound **1** showed specific rotation[13] of −11.2°, which is opposite in sign of the reported specific rotation (+16.3°) of complestatin.[5] Complestatin has been previously converted to chloropeptin I ($[\alpha]_D$ −18.7°) by treatment with hydrochloric acid.[6a,7,12] Similar acid treatment of compound **1** with HCl or TFA produced chloropeptin I ($[\alpha]_D$ −28°)[13] with identical physical, chemical, and ^{1}H and ^{13}C NMR spectral properties including axial chirality and conformation as determined by the comparison of NOESY correlations and molecular models.[6] Like complestatin, the acid hydrolysis of isocomplestatin produced (*R*)-4-hydroxyphenylglycine and (*R*)-3,5-dichloro-4-hydroxyphenylglycine, indicating that compound **1** and complestatin (**2**) are not enantiomers. In addition, we have observed that the slight structural changes in these compounds (cf. chloropeptin I, complestatins A and B) led to significant changes in the ^{1}H NMR shifts of a number of protons located in residues D (H-4 and H-8) and F (H-1α, H-3). This precludes the possibility of epimerization of any chiral centers and leaves the possibility of opposite axial chirality of residues D and F. The different axial chirality of tryptophan between the two compounds may cause a significant difference in the specific rotation without causing much difference in the other properties, including ^{1}H NMR shifts. This could explain the existence of the small difference of the ^{1}H NMR shifts of the H-3 of residue F (vide infra). The NOESY correlations and ChemDraw 3D modeling led to the elucidation of complete stereochemistry including axial chirality of compound **1**, as illustrated in Figure 3. Compound **1** revealed the NOESY correlations between Trp H-2 and Trp-H-2′, and Trp-H-3β and H-5′, as well as H-6′ and H-8 (residue D), indicating that indole nitrogen (tryptophan N-1′) is pointing down, similar to chloropeptin I. The H-3α appeared as a doublet of doublets at δ 2.87 (J = 12, 2.4 Hz) and the H-3β appeared as an apparent triplet at δ 3.44 (J = 12 Hz) in the ^{1}H NMR spectrum of compound **1**, whereas both of these protons appeared at δ 2.89 in complestatin (**2**).[5d] The J values of the protons at C-3 of **1** are consistent with the dihedral angles ($\Phi_{H-2\alpha,H-3\beta}$ = −176°, and $\Phi_{H-2\alpha,H-3\alpha}$ = 60°) of the isomer shown in Figure 3 and would be inconsistent with the dihedral angles ($\Phi_{H-2\alpha,H-3\beta}$ = −80°, and $\Phi_{H-2\alpha,H-3\alpha}$ = 36°) of the opposite axial isomer. Therefore, on the basis of all the data structure **1** is assigned to isocomplestatin.[14]

Our observation of acid-catalyzed rearrangement[9] of the complestatin ring system to the chloropeptin ring system has been confirmed with model systems.[8c] The rearrangement outcome is unaffected by the stereochemistry of the α-carbon of tryptophan, but corresponding acyclic systems do not undergo rearrangement.[8c]

Complestatin A (4) and Complestatin B (5). ESI mass spectral analysis of complestatin A (**4**) and complestatin B (**5**) showed pseudomolecular ions at m/z 1041 $(M + H)^+$ and m/z 1057 $(M + H)^+$, respectively. High-resolution ESI-FTMS analyses of the molecular ion of both compounds gave exact masses of 1041.1137 and 1057.1081, analyzing for $C_{61}H_{46}Cl_6N_7O_{16}$+H and $C_{61}H_{46}Cl_6N_7O_{17}$+H, respectively. These formulas consist of one and two extra oxygen atoms in complestatin A and B, respectively, compared to complestatin, isocomplestatin, and chloropeptin I. UV spectra of isocomplestatin, complestatin A, and complestatin B were qualitatively identical. The ^{1}H and ^{13}C NMR spectra (Table 1) of isocomplestatin (**1**), complestatin A (**4**), and complestatin B

Table 1. ^{1}H (600 and 500 MHz) and ^{13}C NMR (125 MHz) Assignment of Isocomplestatin (**1**), Complestatin A (**4**), and Complestatin B (**5**) in DMSO-d_6[a]

	isocomplestatin (**1**)		complestatin A (**3**)		complestatin B (**4**)	
position	δ_C	δ_H, *J*, Hz	δ_C	δ_H, *J*, Hz	δ_C	δ_H, *J*, Hz
			Hydroxyphenylglycine (A)			
1	171.4		171.6		171.4	
2	55.8	5.05, d, 6.2	56.0	5.06, d, 6.0	55.8	5.06, d, 6.0
3	127.8		127.9		128.1	
4,8	128.2	7.10, d, 8.5	128.4	7.10, d, 9.0	128.2	7.09, d, 8.4
5,7	115.3	6.76, d, 8.5	115.5	6.74, d, 9.0	115.3	6.75, d, 8.4
6	157.2		157.4		157.2	
NH		8.52, d, 6.5		8.43, d, 6.6		8.42, d, 6.6
			Tyrosine (B)			
1	168.5		168.7		168.5	
2	61.2	5.06, m	61.5	5.06, m	61.3	5.07, m
3	35.1	3.05, m	35.1	3.0, m	35.0	3.04, m
4	134.4		134.3		134.2	
5	130.6	7.18, dd, 8.5, 1	130.7	7.18, dd, 8.5, 1.5	130.5	7.18, dd, 8.4, 1.8
6	121.7	7.06, dd, 8.5, 2.3	121.5	7.15, dd, 8.4, 2.4	121.5	7.14, dd, 8.4, 2.4
7	155.2		156.1		155.8	
8	123.2	6.84, brd, 8.5	122.1	6.76, dd, 8.4, 2.4	122.9	6.78, dd, 8.4, 2.4
9	131.5	7.81, dd, 8.5, 1.5	131.3	7.78, dd, 9.0, 2.0	131.6	7.80, dd, 8.4, 2.4
NCH_3	31.3	2.97, s	31.9	3.0, s	31.2	3.0, s
			3,5-Dichloro-4-hydroxyphenylglycine (C)			
1	169.2		169.5		169.3	
2	51.4	5.19, d, 6.2	51.9	5.18, d, 6.6	51.6	5.19, d, 6.0
3	131.1		131.1		131.2	
4,8	126.9	7.33, s	127.2	7.36, s	127.0	7.37, s
5,7	122.0		122.0		122.0	
6	148.7		148.9		148.4	
NH		8.73, d, 6.0		8.90, d, 6.0		8.91, d, 6.0
			3,4-Dihydroxyphenylglycine (D)			
1	167.6		167.4		167.6	
2	55.0	5.56, d, 8.3	55.3	5.69, d, 7.8	54.9	5.71, d, 9.0
3	126.3		127.2		127.7	
4	110.5	5.46, d, 2.2	113.0	5.76, d, 2.4	112.9	5.76, d, 2.4
5	149.6		149.7		149.5	
6	139.4		140.2		140.6	
7	131.1		130.1		130.5	
8	129.5	5.06, d, 2.0	126.1	5.69, d, 2.4	125.8	5.73, d, 2.4
NH		8.28, d, 9.4		8.61, d, 9.6		8.53, d, 9.0
OH		9.40, s		9.45, s		9.46, s
			3,5-Dichloro-4-hydroxyphenylglycine (E)			
1	169.9		170.9		170.6	
2	51.1	5.56, d, 8.6	54.5	5.54, d, 9.0	54.2	5.54, d, 8.4
3	131.9		131.9		130.5	
4,8	126.7	7.28, s	126.4	7.02, s	126.1	7.02, s
5,7	121.7		122.2		121.9	
6	148.2		148.7		148.7	
NH		7.82, d, 8.6		7.14, d, 9.0		7.18, d, 8.4
			Tryptophan (F)			
1	170.5		167.8		166.7	
2	57.0	4.15, ddd, 11, 9, 3	48.2	3.45, t, 8.0	47.3	3.46, m
3	28.3	α: 2.87, dd, 12, 2.4 β: 3.40, t, 12	29.1	3.00, m	35.1	3.00, m
1′	NH	10.87, s	NH	10.6, s	NH	10.5, s
2′	123.6	7.25, d, 2.1	178.9		178.4	
3′	111.8		43.7	3.65, t, 3.9	75.5	
4′	126.3		126.2		128.2	
5′	118.4	7.42, d, 8.4	121.5	7.08, d, 9.0	122.5	7.12, d, 7.8
6′	123.6	6.82, d, 8.4	115.5	6.74, dd, 7.8, 1.2	114.6	6.75, dd, 7.8, 1.2
7′	134.3		127.8		127.0	
8′	114.4	7.23, s	110.9	6.76, d, 1.2	110.9	6.71, d, 1.2
9′	136.2		142.2		140.9	
NH		8.55, brs		9.31, brs		9.35, brs
			3,5-Dichloro-4-hydroxyphenyl Oxo Acid (G)			
1	164.5		164.1		163.8	
2	185.7		183.6		184.0	
3	127.2		127.2		127.2	
4,8	130.5	7.75, s	130.7	7.82, s	130.5	7.83, s
5,7	122.5		122.7		122.0	
6	156.8		156.5		156.5	

[a] Unassigned OH groups each broad singlets, **1**: δ 9.47, 9.85, 10.07; **4**: δ 6.51, 9.47, 9.97, 10.08; **5**: δ 6.19, 9.49, 9.98, 10.09.

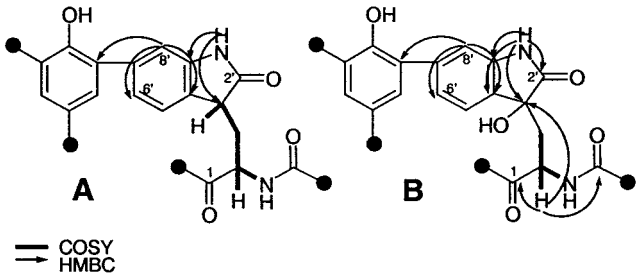

Figure 4. COSY and HMBC correlations of fragments of complestatins A (A) and B (B).

(**5**) were essentially identical except for the signals originating from the tryptophan residue (F) and downfield shift of aromatic protons of residue D. This indicated that the extra oxygen(s) were located in the tryptophan residue (F) in both of these compounds. The ^{13}C NMR spectrum of complestatin A (**4**) revealed a signal for a carbonyl group at δ 178.9 and a methine carbon at δ 43.7. Complestatin B (**5**) exhibited a signal for a carbonyl group at δ 178.9 and an oxygenated quaternary carbon at δ 75.5 instead of olefinic carbons at δ 123.6 and 111.8 for C-2′ and C-3′ of tryptophan, respectively. These assignments were confirmed by HMBC correlations of NH-1′ to C-3′ in complestatin A (Figure 4A) and NH-1′ to C-2′ and C-3′, and H-2 to C-3′, in complestatin B (Figure 4B).

Stereochemistry of Complestatins A and B. The NOESY (mix = 300 ms) spectrum of complestatin A (**4**) exhibited essentially identical correlations to that observed for isocomplestatin and chloropeptin I[6c] except for the residue F and thus indicated identical backbone stereochemistry of amino acids. Within the residue F, H-3′ showed NOESY correlation to H-5′, and NH-1′ showed correlation to H-8′. Similar NOESY correlations were also observed for complestatin B. Extensive NOESY and molecular modeling (see Figure 5, which shows the models of 3′*R* and 3′*S* stereoisomers of complestatin B) studies failed to define the stereochemistry at the new asymmetric carbon of the tryptophan moiety. The conformation of complestatins A and B appeared to be similar to isocomplestatin except for the oxidized tryptophan due to relatively free rotation caused by the sp^3 carbon.

Hydrolysis of Chloropeptin I. Brief accounts of the selective hydrolysis of chloropeptin I (**3**) to fragments **6**−**9** (Figure 6) have already been described.[10] The experimental details of the hydrolysis, characterization of a new hydrolytic product, and the biological activities of the fragments are described here. The terminal hydroxyphenyl glycine of **3** was selectively hydrolyzed by heating with trifluoroacetic acid at 60 °C to quantitatively give compound **6**. Heating of chloropeptin I (**3**) in a vacuum hydrolysis tube under inert atmosphere with a mixture of acetic acid−HCl−thioglycolic acid gave compound **7**. Prolonged heating of the reaction mixture led to the formation of compounds **8**−**10** (Figure 6).[10] The progress of the reaction was monitored by analytical HPLC. The hydrolytic reaction led to the formation of compound **6** in less than 1 h, which was subsequently converted to compound **7**. The formation of the latter compound was maximized at about 2 h and upon further heating was converted to compounds **8**−**10**. The time course of the reaction has been described previously.[10] Compound **9** was isolated from the reaction mixture and is characterized here for the first time. Although chloropeptin I has good solubility in methanol and many other polar organic solvents, its solubility in an aqueous environment was poor even at high temperature. Acetic acid as a cosolvent was helpful in the dissolution of chloropeptin I. Thioglycolic acid was useful in preventing decomposition of tryptophan.

The cleavage of the terminal 4-hydroxymethyl glycine led to the *cis*−*trans* isomerization of the tertiary *N*-methyl amide in compounds **6** and **7**. These compounds each showed two HPLC peaks that could be easily separated by preparative HPLC. However, the HPLC analysis of the fractions, supposedly containing a single compound, revealed that the fractions consisted of the same two compounds present in the starting

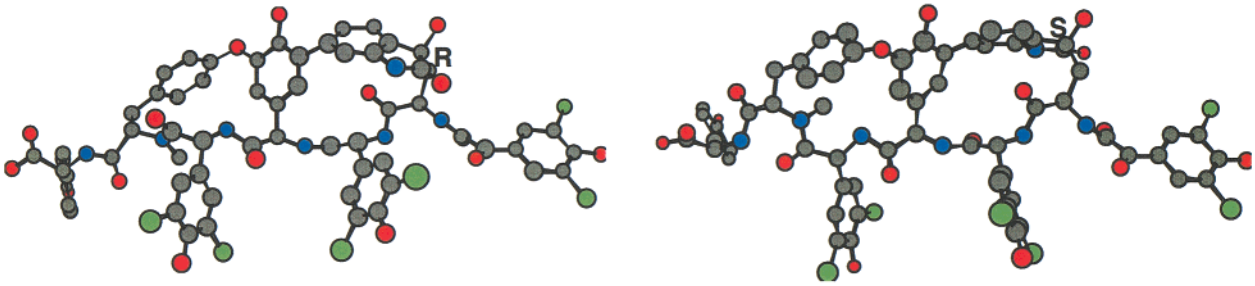

Figure 5. ChemDraw 3D generated models of complestatin B (**5**) with 3′*R* and 3′*S* stereochemistry.

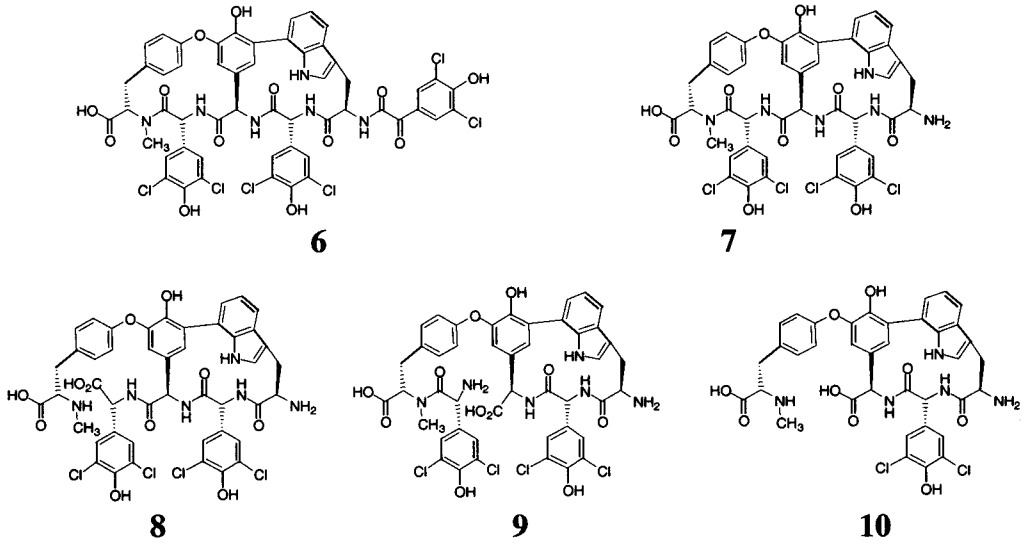

Figure 6. Structures of hydrolytic fragments of chloropeptin I.

Table 2. Biochemical and Antiviral Activities of Isocomplestatin and Related Compounds (**1**−**10**)

compound	HIV-1 integrase (IC_{50} μM)		HIV-1 viral spread (IC_{95} μM)
	coupled	strand transfer	
1	0.2	4	5
3	0.4	5	5
4	0.8	12.5	ND[a]
5	1.7	12.5	ND
6	0.6	8	5
7	0.5	6	7.5
8	3.8	45	ND
9	7.8	86	ND
10	28	>100	ND

[a] ND (not determined)

Table 3. Other Activities of Isocomplestatin

assay	IC_{50} (μM)
HIV disintegration	0.5
3′-end processing	0.2
integration	3.0
rFIV	0.5
rSIV	0.10
rAMV	0.25
rHIV (PIC)	3.0
rHIV	0.2
gp120-CD45	2.0

mixture due to isomeric equilibration. The rate of the isomeric equilibration was different for each isomer and was solvent dependent. For example, in aqueous CH_3CN + 0.1% TFA equilibration of the faster eluting isomer was more rapid than that of the slower eluting isomer. In less than 20 min, the fraction containing the faster eluting isomer was equilibrated to a ∼50:50 mixture consisting of both isomers, but it took more than 1 h for the same equilibration of the slower eluting isomer. The stability and the rate of the equilibration were reversed in methanol. For example in the case of compound **7**, the slower eluting isomer was completely converted (>90%) to the faster eluting isomer after 24 h in MeOH, as evidenced by HPLC. The ^{1}H NMR spectra of both compounds **6** and **7** in DMSO-d_6 showed a 2:1 isomeric ratio even at higher temperatures (50−100 °C). However the preponderance of only one of the two isomers was observed when the ^{1}H NMR spectra were recorded in THF-d_8 in the presence of saturating amounts of LiCl. This phenomenon of LiCl-induced change in isomeric population is consistent with the observation originally made for *N*-methylated compounds, i.e., cyclosporin.[15] The exchangeable hydroxy protons were broadened and were present throughout the spectrum recorded in THF-d_8. The ^{1}H NMR spectrum of compound **8** also showed the presence of two major isomers due to *cis*−*trans* isomerization of the tertiary *N*-methyl amide group; however the isomers were not separable by HPLC. It is interesting to note that in the presence of terminal 4-hydroxyphenylglycine this *cis*−*trans* isomerization of *N*-methyl amide is not observed (cf. isocomplestatin, complestatins, and chloropeptin I).

Biological Activity. Isocomplestatin was isolated following bioassay-guided fractionation using an in vitro HIV-1 integrase assay that recapitulates the staged process of viral integration.[4] Subsequently, isocomplestatin and all of the other compounds were evaluated in a number of related biological and biochemical assays including the HIV-1 multiple cycle assay for viral replication (viral spread), and the data are shown in Tables 2 and 3. Complestatin (**2**) and chloropeptin I (**3**) were reported[6a,b] to inhibit the binding of gp120 to CD4 with IC_{50} values of 2 and 1.3 μM, respectively. Like complestatin, isocomplestatin was shown to affect the formation of syncitia at μM levels in cell culture. Although this is higher than the concentration required to inhibit HIV-1 replication (vide infra), we cannot exclude the possibility that the antiviral activity of isocomplestatin is all or in part due to the effect on the HIV-1 envelope GP120/CD4 receptor interaction. Isocomplestatin (**1**), chloropeptin I (**3**), and the hydrolytic fragments (**6** and **7**) were evaluated in the HIV-1 viral spread assay.[2e] These compounds exhibited IC_{95} values of 5, 5, 5, and 7.5 μM, respectively (Table 2). Isocomplestatin (**1**) in the same assay showed an IC_{50} value of 0.2 μM.

HIV-1 Integrase Activity. Isocomplestatin (**1**) inhibited in vitro HIV-1 integrase coupled and strand transfer activities with IC_{50} values of 0.2 and 4.0 μM, respectively. Chloropeptin I (**3**), complestatin A (**4**), and complestatin B (**5**) were less active than isocomplestatin and exhibited IC_{50} values of 0.4−1.7 and 5−12.5 μM in the coupled and strand transfer assays, respectively. The comparable activities of these compounds are not surprising due to their structural similarity. This was further confirmed from the molecular model comparison of these compounds. The molecular models (ChemDraw 3D) of all four compounds were highly similar, except for a slight and expected deviation of the tryptophan-containing 16-membered ring of chloropeptin I. The hydrolytic fragments (**6** and **7**) with intact macrocyclic rings retained almost all of the activities and exhibited IC_{50} values of 0.6, 0.3 μM and 8.0, 6.0 μM in coupled and strand transfer assays, respectively. However, opening of one of the macrocyclic rings caused a significant decrease in the integrase activity (see compounds **8**−**10** in Table 2). Isocomplestatin inhibits preintegration complexes from HIV-1-infected cells with an IC_{50} comparable to that observed for inhibition of strand transfer using complexes assembled on the viral donor DNA with recombinantly expressed integrase. This is similar to what has been observed previously for the diketoacid inhibitors of integrase.[2e] The observation that isocomplestatin is active both in strand transfer and against HIV-1 preintegration complexes suggests this compound may be an alternative platform on which to design novel strand transfer inhibitors.

It should be noted that in contrast to the diketoacid inhibitors, which are much weaker inhibitors of 3′ processing than strand transfer, isocomplestatin and the derivatives reported herein inhibit 3′ processing with a characteristically 10-fold better IC_{50} than the IC_{50} measured in strand transfer (Table 2). These data suggest that isocomplestatin may preferentially bind to the uncomplexed enzyme, unlike the diketoacid, which requires binding the viral DNA end.[2e] Also, unlike the diketoacid, isocomplestatin inhibits the HIV-1 disintegration activity of both the intact protein and the catalytic core domain of integrase (amino acids 50−212) with comparable potency (IC_{50} 0.5 μM); thus, isocomplestatin binds to the core domain of integrase. Isocomplestatin inhibits the integrase protein from several related retroviruses. Isocomplestatin inhibited recombinant feline immunodeficiency virus with an IC_{50} value of 0.5 μM and was a more potent inhibitor of recombinant simian immunodeficiency virus, with an IC_{50} value of 0.1 μM.

In conclusion we report absolute stereochemistry and efficient processes for isolation of isocomplestatin, which can be implemented from a gram to an industrial scale. We have comprehensively evaluated these compounds against HIV-1 integrase and various other retroviral targets. In addition, we have reported two new oxidized congeners of isocomplestatin, which has doubled the number of the members of this small family of bicyclic hexapeptides. Selective hydrolysis of chloropeptin I and the retention of most of the biological activity by the bicyclic fragments shed light on the structure−activity relationship of this class of compounds and may lead to better inhibitor design and understanding of HIV-1 integrase target.

Experimental Section

General Experimental Procedures. Amberchrom CG161, a styrenic-based resin, was obtained from TosoHaas Corporation. HP20 and SP207 were obtained from Mitsubishi Chemical Company. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. All NMR spectra were recorded on Varian Inova 400, 500, or 600 MHz instruments operating at 400, 500, and 600 MHz for ^{1}H and 100, 125, and 150 MHz for ^{13}C nuclei. An HP1100 was used for analytical HPLC. LCMS was performed on a Thermo Quest LCQ instrument using electrospray ionization (ESI) or atmospheric pressure

chemical ionization (APCI). High-resolution mass spectral analysis was performed on a Thermo Quest FTMS using electrospray ionization.

Fermentation of MA7234. *Streptomyces* sp. (MA7234) was grown on an ISP Medium2 plate (DIFCO, Detroit, MI) for 7 days at 28 °C. Growth was scraped from the plate and inoculated into a 250 mL baffled flask containing 50 mL of ATCC-2 media [soluble starch (20 g); dextrose (10 g); NZ amine E (5 g); Difco beef extract (3 g); Difco bacto peptone (5 g); Fidco yeast extract (5 g); $CaCO_3$ (1 g); in 1 L distilled water; pH was 7.0 prior to the addition of calcium carbonate]. Flasks were shaken at 220 rpm for 4 days at 28 °C, and 1.5 mL of the ATCC culture broth was transferred to 250 mL nonbaffled production flasks containing 45 mL of KHC media containing dextrin (20 g); β-cyclodextrin (10 g); primary yeast (10 g); tomato paste (20 g); $CoCl_2-6H_20$ (0.005 g); in 1 L distilled water at pH 7.2. The inoculated flasks containing production media were shaken at 220 rpm for 8 days at 28 °C. The culture broths were pooled together and extracted as described below.

Bioassay-Guided Isolation of Isocomplestatin. Fermentation broth (0.5 L) was extracted with methyl ethyl ketone (0.5 L), and the extract was filtered and evaporated to produce 300 mg of residue. The residue was dissolved in 100 mL of methanol and was charged onto a 1.5 L Sephadex LH-20 column in methanol. Elution of the column with methanol afforded the HIV-1 integrase active fractions in 1.2 column volumes. The active fractions were concentrated under reduced pressure to produce 46 mg of a brown gum. A 20 mg portion of the gum was purified on a reverse-phase Zorbax RX C-8 HPLC column (22.4 × 250 mm) eluting with aqueous CH_3CN containing 0.1% TFA at a flow rate of 10 mL/min. The column was initially eluted with a 50 min gradient of 10−40% CH_3-CN followed by a 10 min gradient to 70% CH_3CN. Activity was concentrated in a broad peak eluting at 76 min. Lyophilization of the combined active fractions gave isocomplestatin (**1**, 12.0 mg, 50 mg/L) as a buff-colored powder. The UV, ^{13}C NMR, and mass spectral data were identical to complestatin.[5]

Initial Large-Scale Isolation of Isocomplestatin and Isolation of Complestatins A and B Using Acidic Conditions. A 6 L fermentation grown in liquid (pH = 7.6) media was filtered in order to separate the cells from the extract. The filtrate (4 L) was acidified to pH 2.0 by addition of 4 N HCl and extracted with ethyl acetate (2 × 4 L). The ethyl acetate extract was concentrated to dryness to give 1.5 g of residue, which was purified on a 2 L Sephadex LH-20 column in methanol. The column was eluted with three column volumes of methanol followed by two column volumes of a 1:1 mixture of methanol−acetone to afford 180 mg of a mixture of isocomplestatin and related compounds as a buff-colored powder. Analysis of the powder by HPLC (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3CN containing 0.1% TFA, 1 mL/min) indicated it to be an approximately 10:1:1:1 mixture of isocomplestatin (**1**, t_R = 8.8 min), chloropeptin I (**3**, t_R = 9.6 min), complestatin A (**4**, t_R = 5.7 min), and complestatin B (5, t_R = 4.3 min). A 53 mg portion of the residue was chromatographed on a reverse-phase Zorbax SB CN HPLC column (22.4 × 250 mm) using a gradient of aqueous CH_3CN + 0.1% TFA at a flow rate of 10 mL/min. The column was eluted with 20% of CH_3CN for 12 min, then a linear gradient to 40% in 10 min, held for 10 min, followed by a linear gradient to 60% of CH_3-CN in 10 min to give **1** (5 mg containing ~10% of chloropeptin I, **3**), **4** (3.5 mg), and **5** (4 mg) as buff powders.

The cells were extracted with 70% aqueous acetone (2 × 1.5 L), and the extract was concentrated under reduced pressure to a volume of 1.2 L, cooled, and acidified to pH 2.0 by addition of 4 N HCl. The acidified solution was extracted with EtOAc (2 × 1.2 L), and the EtOAc extract was concentrated under reduced pressure to a volume of 150 mL and extracted twice with 150 mL of 5% aqueous $NaHCO_3$ solution. The sodium bicarbonate extracts were pooled, cooled, acidified to pH = 2.0, and re-extracted with EtOAc (2 × 250 mL). The EtOAc extract was concentrated to dryness, shaken in methanol, and then centrifuged to give 55 mg of a dark brown powder consisting of a mixture of isocomplestatin and chloropeptin I.

Isolation of Isocomplestatin (1) by Amberchrom Chromatography at pH 2.5. A 10 L fermentation was filtered, and the cells were extracted twice with 4 L and once with 2 L of 70% aqueous acetone by stirring at room temperature overnight. The pH of the extract was ~7.0. The quantitative HPLC analysis of the acetone extract (9 L) revealed the presence of 2.5 g of isocomplestatin. A small portion (100 mL) of extract was concentrated to a small volume and acidified with 4 N HCl to pH 2.5. The acidification resulted in the formation of a precipitate that was collected by filtration to give isocomplestatin (9 mg). The filtrate was charged onto 10 mL of Amberchrom preequilibrated at pH 2.5 with 30% aqueous acetone. The column was washed with 30% and 50% aqueous acetone at pH 2.5 and eluted with 70% aqueous acetone to give 12.5 mg of a 2:1 mixture of isocomplestatin and chloropeptin I.

Isolation of Isocomplestatin Free of Chloropeptin I. A 13 L fermentation of the culture was filtered through Celite to give 9 L of filtrate (pH = 8.4). Mycelia were separated and extracted five times by shaking with 2 L each of 70% aqueous acetone to give a combined extract of 9 L. A quantitative HPLC analysis (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3-CN + 0.1% TFA, 1 mL/min) indicated the presence of ~1.3 and 1.1 g (>90%) of isocomplestatin (t_R = 8.8 min) in extracts A and B, respectively, while chloropeptin I (t_R = 9.6 min) was not detectable.

(1) By Amberchrom Chromatography at pH >8. Eight liters of filtrate (pH = 8.4) was charged to a 1 L, thoroughly washed Amberchrom column at a flow rate of 15 mL/min. The column was washed with 15 L of water followed by 4 L each of 10% and 20% aqueous methanol. Isocomplestatin was eluted with 66% aqueous acetone (0.6 L) and finally with acetone (1 L). All eluates containing isocomplestatin were combined and concentrated under reduced pressure to yield a mostly aqueous solution, which was lyophilized to give salts of isocomplestatin as a brown powder (1.1 g). The fractions eluting with 50−66% acetone contained complestatins A (**4**) and B (**5**).

The aqueous acetone extract was concentrated to a total volume of ~5 L and diluted with 4 L of water. The pH of this solution was adjusted to 8.5 by addition of 10% aqueous sodium bicarbonate and chromatographed on a similar Amberchrom column to give 1.4 g of isocomplestatin salt that was contaminated with large amounts of fatty compounds (^1H NMR).

(2) By SP207 Chromatography at pH >8. One hundred milliliters (containing 14.4 mg of isocomplestatin) of filtrate (pH = 8.4) was charged on a 60 mL SP207 column. The column was washed with water (400 mL) and 100 mL each of 10% and 20% methanol, and isocomplestatin salt was eluted with 50% aqueous methanol (80 mL) followed by 160 mL each of 50% aqueous acetone and acetone. The combined solution was concentrated under reduced pressure to yield mostly aqueous solution that was lyophilized to give 21 mg (containing 13 mg, 90% yield) of isocomplestatin salts.

(3) By HP20 Chromatography at pH >8. One hundred milliliters of the filtrate was charged onto a 60 mL HP20 column, and the chromatography was repeated in an identical manner as described for SP207 to give 19 mg (containing 13 mg, 90% yield) of isocomplestatin salts.

Regeneration of Isocomplestatin from Isocomplestatin Salts. Isocomplestatin salts (1.1 g) prepared from the filtrate by Amberchrom chromatography were dissolved in 50 mL of methanol and acidified by addition of 30 mL of 10% aqueous citric acid. This led to the formation of a precipitate of salt-free isocomplestatin. The precipitate was collected on a scintered glass funnel and washed with 10% aqueous citric acid (50 mL), water (50 mL), methanol (100 mL), ethyl acetate (100 mL), and finally hexane (100 mL). The resulting powder was dried in a vacuum desiccator overnight to afford 1.03 g (two step yield ~90%) of highly pure (HPLC, LCMS, ^1H NMR) isocomplestatin (**1**) as a brown solid.

A similar regeneration process produced 0.5 g of isocomplestatin from 1.4 g of salt isolated from the aqueous acetone extract and 12 mg each from SP207 and HP20 chromatogra-

phy. The fatty impurities present with isocomplestatin salts were easily removed during the organic solvent washes of the regeneration process and led to highly pure isocomplestatin. Small amounts of complestatins A and B that were present with isocomplestatin were also washed out due to their better solubility in methanol.

Isocomplestatin (1): t_R 8.8 min (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3CN + 0.1% TFA, 1 mL/min); $[\alpha]_D^{22}$ −11.2° (*c*, 1.6, DMSO); $[\alpha]_D^{22}$ −60° (*c*, 0.25, CH_3OH−0.01 N NaOH, 2:1) {lit. $[\alpha]_D^{26}$ +24.5° (*c*, 0.13, CH_3OH−0.01 N NaOH, 2:1)};[5d] $[\alpha]_D^{26}$ +16.3° (*c*, 1.6, DMSO);[6a] UV λ_{max} (CH_3CN−H_2O + 0.1% TFA): 205, 240, 290 nm; HR-ESI-FTMS (*m/z*) 1326.1155 [(M + H)$^+$, calcd for $C_{61}H_{46}Cl_6N_7O_{15}$ 1326.1183]; ^{1}H and ^{13}C NMR (see Table 1).

Chloropeptin I (3): t_R 9.6 min (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3CN + 0.1% TFA, 1 mL/min); $[\alpha]_D^{22}$ − 84° (*c*, 0.5, CH_3OH); $[\alpha]_D^{22}$ −28° (*c*, 0.5, DMSO) {lit.[6c] $[\alpha]_D^{26}$ −18.7° (*c*, 0.16, DMSO)}; UV λ_{max} (CH_3CN−H_2O + 0.1% TFA): 205, 240, 290 nm; HR-ESI-FTMS (*m/z*) 1326.1161 [(M + H)$^+$, calcd for $C_{61}H_{46}Cl_6N_7O_{15}$ 1326.1183]; ^{1}H and ^{13}C NMR (see ref 7b).

Complestatin A (4): t_R 5.7 min (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3CN + 0.1% TFA, 1 mL/min); $[\alpha]_D^{22}$ +10° (*c*, 0.1, CH_3OH); UV λ_{max} (CH_3CN−H_2O + 0.1% TFA) 205, 240, 290 nm; HR-ESI-FTMS (*m/z*) 1342.1137 [(M + H)$^+$, calcd for $C_{61}H_{46}Cl_6N_7O_{16}$ 1342.1132]; ^{1}H and ^{13}C NMR (see Table 1).

Complestatin B (5): t_R 4.3 min (Zorbax RX C-8, 4.6 × 250 mm, 50% aqueous CH_3CN + 0.1% TFA, 1 mL/min); $[\alpha]_D^{22}$ +20° (*c*, 0.1, CH_3OH); UV λ_{max} (CH_3CN−H_2O + 0.1% TFA) 205, 240, 290 nm; HR-ESI-FTMS (*m/z*) 1358.1120 [(M + H)$^+$, calcd for $C_{61}H_{46}Cl_6N_7O_{17}$ 1358.1081]; ^{1}H and ^{13}C NMR (see Table 1).

Rearrangement of Isocomplestatin to Chloropeptin I. Isocomplestatin (112 mg) was heated in 1 mL of anhydrous TFA at 60 °C for 15 min. The progress of the reaction was analyzed by a reverse-phase HPLC (Zorbax RC C-8, 4.6 × 250 mm, 50% aqueous CH_3CN + 0.1% TFA, flow rate 1 mL/min). The conversion of isocomplestatin (t_R = 8.8 min) to chloropeptin I (t_R = 9.6 min) was complete in less than 15 min. TFA was evaporated under reduced pressure to give homogeneous (by HPLC, ^{1}H NMR, and ^{13}C NMR) chloropeptin I (110 mg). An analytical sample was prepared by reverse-phase preparative HPLC using one of the gradients described earlier followed by lyophilization, giving buff-colored chloropeptin I (**3**). The ^{1}H and ^{13}C NMR spectral data of chloropeptin I were identical to that reported in the literature.[6b]

Hydrolysis of Chloropeptin I with Trifluoroacetic Acid. Isocomplestatin (20 mg) was heated in anhydrous TFA (0.2 mL) at 60 °C for 15 min to give chloropeptin I. The reaction mixture was further heated, and the progress of the reaction was examined by HPLC (Zorbax RC C-8, 4.6 × 250 mm, 10−30% aqueous CH_3CN + 0.1% TFA in 20 min then 30−80% CH_3CN in another 20 min, flow rate 1 mL/min, gradient A). The reaction was mostly complete after 24 h but heated for an additional 3 days to afford exclusively compound **6** (t_R = 38.4 min) and 4-hydroxyphenylglycine (t_R = 5.2 min). Chloropeptin I eluted at 37.5 min in gradient A. TFA was evaporated under reduced pressure, and the residue was chromatographed using a reverse-phase HPLC (Zorbax RX C-8, 22 × 250 mm) and eluted with a 40 min gradient of 40−60% aqueous CH_3CN + 0.1% TFA at a flow rate of 10 mL/min. Two peaks were collected and lyophilized to give 1.5 mg of 4-hydroxyphenylglycine and 10 mg of compound **6** as amorphous powders. Reexamination of the compound **5** by HPLC using gradient A showed two peaks, t_R = 38.4 and 37.2 min, in a ∼1:1 ratio due to *cis*−*trans* isomerization of *N*-methyl tertiary amide. The ratio of the two compounds varied depending on the way the sample was stored and how it was analyzed by HPLC. 4-Hydroxyphenylglycine: $[\alpha]_D^{25}$ −51.4° (*c*, 0.18, 2 N HCl) {lit. $[\alpha]_D^{25}$ for *R*-isomer −156° (*c*, 1, 1 N HCl)[16] and −125°[5d]}. **6**: t_R 8.55 and 9.7 min (Zorbax RX C-8, 4.6 × 250 mm, 55% aqueous CH_3CN + 0.1% TFA, 1 mL/min); ^{1}H NMR (DMSO-d_6, 70 °C, δ) 2.84 (3H, s, *N*-CH_3), 3.00 (3H, m), 3.28 (1H, m), 4.88 (1H, dd, *J* = 11.5, 3 Hz), 4.96 (1H, d, *J* = 5.5 Hz), 5.04 (1H, m), 5.36 (1H, d, *J* = 8.5 Hz), 5.52 (1H, brd, *J* = 9 Hz), 5.57 (1H, d, *J* = 8.5 Hz), 5.72 (2/3H, d, *J* = 2 Hz), 5.82 (1/3H, brs), 5.92 (2/3H, d, *J* = 1.5 Hz), 6.06 (1/3H, brs), 6.78 (1/3H, dd, *J* = 8, 2.5 Hz), 6.92, 6.93 (1H, t, *J* = 7. 5 Hz), 7.03 (1/3H, dd, *J* = 8, 2 Hz), 7.05 (1/3H, d, *J* = 7 Hz), 7.10 (1/3H, d, *J* = 8 Hz), 7.15 (2/3H, dd, *J* = 8, 2 Hz), 7.18 (2/3H, dd, *J* = 8, 2 Hz), 7.23 (2/3H, d, *J* = 9 Hz), 7.25 (2/3H, d, *J* = 9 Hz), 7.26 (1H, s), 7.39 (4H, s), 7.59 (1H, dd, *J* = 9, 2 Hz), 7.68 (1H, dd, *J* = 8.5, 2 Hz), 7.87, 7.88 (2H, s), 7.93 (1H, d, *J* = 9 Hz), 8.06 (1H, d, *J* = 8.5 Hz), 8.53 (1/3H, d, *J* = 6.5 Hz), 8.63 (2/3H, d, *J* = 8.5 Hz), 9.98 (1/3H, brs), 10.23 (2/3H, brs); ^{1}H NMR (THF-d_8 + saturating LiCl, δ) 3.06 (3H, s, *N*-CH_3), 3.13 (3H, m), 3.69 (1H, m), 4.65 (1H, m), 5.27 (1H, d, *J* = 6.0 Hz), 5.69 (1H, m), 5.79 (1H, d, *J* = 9 Hz), 5.81, 5.97 (1H, d, *J* = 2.5 Hz), 6.09, 6.20 (1H, d, *J* = 2.5 Hz), 6.35 (1H, d, *J* = 9.5 Hz), 6.81 (1H, dd, *J* = 8.5, 2.0 Hz), 6.85 (1H, t, *J* = 7.5 Hz), 7.14 (1H, d, *J* = 7.0 Hz), 7.21 (1H, d, *J* = 8.5, 2.5 Hz), 7.31 (1H, d, *J* = 8.5 Hz), 7.32 (1H, brd, *J* = 8 Hz), 7.42 (1H, brs), 7.51 (2H, s), 7.78 (2H, s), 7.88 (2H, s), 7.99 (1H, brd, *J* = 8 Hz), 8.19 (2H, d, *J* = 6.5 Hz), 10.4 (1H, brs); HR-ESI-FTMS (*m/z*) 11770731 [(M + H)$^+$, calcd for $C_{53}H_{39}Cl_6N_6O_{13}$ 1177.0706].

Mineral Acid Hydrolysis of Chloropeptin I. A solution of chloropeptin I (100 mg) in acetic acid (2 mL), 8 N HCl (0.5 mL), and thioglycolic acid (0.1 mL) was added to a vacuum hydrolysis tube, degassed by pulling vacuum, and purged with nitrogen. The solution was heated at 110 °C for 24 h and was allowed to cool to room temperature. Solvents were removed under reduced pressure to give a powder, which was dissolved in methanol and chromatographed on a Primesphere 5μ C-8 (22 × 250 mm) column and eluted with an aqueous CH_3CN + 0.1% TFA gradient (10% aqueous CH_3CN for 10 min, 10 to 70% in 60 min) at a flow rate of 8 mL/min. The fractions were analyzed by analytical HPLC, and similar fractions were mixed and lyophilized to give the following four major fractions: A (5−10 min, 21.4 mg), compound **10** (34−40 min, 2.2 mg), B (41−49 min, 28.4 mg), C (50−59 min, 30.1 mg). Fraction A was rechromatographed on the same column and eluted with 20% aqueous CH_3CN + 0.1% TFA for the first 20 min followed by a 20 min gradient to 45% at a flow rate of 8 mL/min. Lyophilization of the fractions gave TFA salts of *N*-methyl-4-hydroxyphenylglycine (7−11 min, 10.1 mg), 4-hydroxyphenylglycine (12−14 min, 5.3 mg [HR-ESI-FTMS (*m/z*) 168.0664 (M + H)$^+$, calcd for $C_8H_{10}NO_3$+H 168.0661]), 3,5-dichloro-4-hydroxyphenylglycine (25−32 min, 3 mg, $[\alpha]^{25}_D$ −10° (*c*, 0.1, 4 N HCl), reported $[\alpha]^{25}_D$ for *R*-isomer −81.6°),[5d] 4-hydroxyphenylglycine methyl ester (33−38 min, 1.5 mg), and compound **10** (63−65 min, 0.3 mg) all as amorphous powders. Fraction B was similarly chromatographed eluting with 20% aqueous CH_3CN + 0.1% TFA for the first 10 min followed by a 60 min gradient to 50% CH_3CN but held at 35% at 30th min onward at a flow rate of 8 mL/min. The like fractions were combined and lyophilized to give compound **10**·TFA (31−32 min, 3.3 mg), 3,5-dichloro-4-hydroxyphenyl oxo-acid (33−35 min, 6 mg), compound **9**·TFA (40 min, 0.8 mg), and compound **8**·TFA (41−42 min, 1.0 mg) as amorphous powders. Fraction C was accordingly chromatographed eluting with 20% aqueous CH_3CN + 0.1% TFA for the first 10 min followed by a 70 min gradient to 45% CH_3CN at a flow rate of 8 mL/min. The fractions eluting from 67 to 71 and 76 to 78 min were combined to give the two interconvertible isomers of compound **7**·TFA (5.1 mg) as a buff powder. **7**: t_R 7.0 and 9.5 min (Zorbax RX C-8, 4.6 × 250 mm, 40% aqueous CH_3CN + 0.1% TFA, 1 mL/min); ^{1}H NMR (DMSO-d_6, 100 °C, δ) 2.86 (3H, s, *N*-CH_3), 3.00 (3H, m), 3.28 (1H, m), 4.39 (1H, m), 4.79, (1H, dd, *J* = 11.5, 3 Hz), 5.33 (2/3H, d, *J* = 8.5 Hz), 5.36 (1H, d, *J* = 9 Hz), 5.48 (1/3H, d, *J* = 9 Hz), 5.51 (1/3H, m), 5.52 (1/3H, d, *J* = 9 Hz), 5.56 (2/3H, d, *J* = 9 Hz), 5.74 (2/3H, d, *J* = 1.5 Hz), 5.77 (1/3H, d, *J* = 1.5 Hz), 5.89 (2/3H, d, *J* = 1.5 Hz), 6.05 (1/3H, brs), 6.77 (1H, dd, *J* = 8, 2.5 Hz), 6.95, 6.96 (1H, t, *J* = 7. 5 Hz), 7.03 (1/3H, dd, *J* = 8, 2 Hz), 7.09 (2/3H, d, *J* = 7 Hz), 7.12 (1/3H, d, *J* = 8 Hz), 7.15 (2/3H, d, *J* = 7 Hz), 7.18 (2/3H, dd, *J* = 8, 2 Hz), 7.23 (1/3H, s), 7.24 (2/3H, s), 7.25 (1H, brs), 7.28, 7.29 (2H, s), 7.49 (1H, dd, *J* = 9, 2 Hz), 7.68 (1H, dd, *J* = 8.5, 2 Hz), 7.97 (2/3H, d, *J* = 8.5 Hz), 8.02 (1/3H, d, *J* = 9 Hz), 8.19 (2/3H, d, *J* = 8.5 Hz), 8.22 (1/3H, d, *J* = 9 Hz), 8.40 (2/3H, d, *J* = 6 Hz), 8.47 (1/3H, d, *J* = 8.5 Hz), 9.96(1/3H, brs), 10.23 (2/3H, brs); ^{1}H NMR (THF-d_8 + saturating LiCl, δ) 3.03

(3H, m), 3.07 (3H, s, *N*-CH_3), 3.57 (1H, m), 5.12 (1H, brm), 5.37 (1H, brm), 5.88 (1H, m), 5.90 (1H, d, 9.5 Hz), 5.95 (1H, d, J = 9 Hz), 5.97 (1H, d, J = 2.0 Hz), 6.09 (1H, d, J = 2.0 Hz), 6.32 (1H, d, J = 9.0 Hz), 6.82 (1H, dd, J = 8.0, 2.0 Hz), 6.92 (1H, t, J = 8 Hz), 7.09 (1H, d, J = 7.0 Hz), 7.15 (2H, dd, J = 8, 2 Hz), 7.20 (1H, d, J = 7 Hz), 7.22 (1H, dd, J = 8, 2 Hz), 7.34 (1H, d, J = 7.5 Hz), 7.38 (1H, brs), 7.60 (2H, s), 7.88 1H, brs), 7.90 (2H, s), 9.96 (1H, brs); HR-ESI-FTMS (*m*/*z*) 961.1330 [(M + H)$^+$, calcd for $C_{45}H_{37}Cl_4N_6O_{10}$ 961.1325]. **8**: t_R 8.6 min (Zorbax RX C-8, 4.6 × 250 mm, 30% aqueous CH_3CN + 0.1% TFA, 1 mL/min); ^{1}H NMR (DMSO-d_6, δ) Tyr (B), 2.50 (3H, brs, *N*-CH_3), 3.04 (2H, m, H_2-3), 3.82 (1H, m, H-2), 6.92 (2H, d, J = 8 Hz, H-6,8), 7.21 (2H, d, J = 8.4 Hz, H-5,9); dichlorohydroxyphenyl glycine (C), 5.06 (1H, d, J = 8 Hz, H − 2), 7.25 (2H, s, H-4,8), 9.07 (1H, d, J = 7.6 Hz, NH); dihydroxyphenyl glycine (D), 5.55 (1H, d, J = 8.8 Hz, H-2), 5.95 (1H, brs, H-4), 6.96 (1H, brs, H-8), 8.60 (1H, d, J = 9.2 Hz, NH); dichlorohydroxyphenyl glycine (E), 5.43 (1H, d, J = 8.4 Hz, H-2), 7.30 (2H, s, H-4,8), 8.35 (1H, d, J = 7.6 Hz, NH); Trp (F), 2.99 (1H, m, H-3), 3.23 (1H, m, H-3), 4.39 (1H, m, H-2), 6.94 (1H, t, J = 8 Hz, H-6), 7.01 (1H, d, J = 7.2 Hz, H-7), 7.26 (1H, d, J = 9.2 Hz, H-5), 7.48 (1H, brs, H-2), 10.52 (1H, brs, H-1); HR-ESI-FTMS (*m*/*z*) 979.1442 [(M + H)$^+$, calcd for $C_{45}H_{39}Cl_4N_6O_{11}$ 979.1431). **9**: t_R 7.3 min (Zorbax RX C-8, 4.6 × 250 mm, 30% aqueous CH_3CN + 0.1% TFA, 1 mL/min); ^{1}H NMR (DMSO-d_6, δ) chemical shifts are reported as an average of the two *cis*−*trans* tertiary *N*-methyl amide isomers, Tyr (B), 2.48 (3H, brs, *N*-CH_3), 3.01 (2H, m, H_2-3), 3.55 (1H, m, H-2), 6.85 (2H, d, J = 8 Hz, H-6,8), 7.17 (2H, d, J = 8.4 Hz, H-5,9); dichlorohydroxyphenyl glycine (C), 5.08 (1H, brs, H-2), 7.22 (2H, s, H-4,8), 8.83 (1H, brs, NH); dihydroxyphenyl glycine (D), 5.46 (1H, d, J = 9.2 Hz, H-2), 6.05 (1H, brs, H-4), 6.62 (1H, brs, H-8), 8.69 (1H, d, J = 8.0 Hz, NH); dichlorohydroxyphenyl glycine (E), 5.35 (1H, d, J = 8.0 Hz, H-2), 7.30 (2H, s, H-4,8), 8.56 (1H, d, J = 8.0 Hz, NH); Trp (F), 2.99 (1H, m, H-3), 3.23 (1H, m, H-3), 4.39 (1H, m, H-2), 6.94 (1H, t, J = 8 Hz, H-6′), 7.01 (1H, d, J = 7.2 Hz, H-7′), 7.27 (1H, d, J = 8.4 Hz, H-5), 7.47 (1H, brs, H-2′), 10.61 (1H, brs, H-1′); HR-ESI-FTMS (*m*/*z*) 979.1444 [(M + H)$^+$, calcd for $C_{45}H_{39}Cl_4N_6O_{11}$ 979.1431).

10: t_R 5.6 min (Zorbax RX C-8, 4.6 × 250 mm, 40% aqueous CH_3CN + 0.1% TFA, 1 mL/min); ^{1}H NMR (DMSO-d_6, δ) Tyr (B), 2.48 (3H, brs, *N*-CH_3), 3.06 (2H, m, H_2-3), 3.77 (1H, m, H-2), 6.96 (2H, d, J = 8 Hz, H-6,8), 7.24 (2H, d, J = 8.4 Hz, H-5,9); dihydroxyphenyl glycine (D), 5.48 (1H, d, J = 8.8 Hz, H-2), 6.07 (1H, d, J = 2 Hz, H-4), 6.93 (1H, d, J = 2 Hz, H-8), 8.68 (1H, d, J = 8.4 Hz, NH); dichlorohydroxyphenyl glycine (E), 5.25 (1H, d, J = 8.4 Hz, H-2), 7.35 (2H, s, H-4,8), 8.53 (1H, d, J = 8.8 Hz, NH); Trp (F), 3.02 (1H, t, J = 12.8 Hz, H-3), 3.26 (1H, dd, J = 15.2, 4.8 Hz, H-3), 4.44 (1H, dd, J = 11.6, 6 Hz, H-2), 6.94 (1H, t, J = 8 Hz, H-6′), 7.03 (1H, d, J = 6.8 Hz, H-7′), 7.27 (1H, d, J = 7.6 Hz, H-5), 7.47 (1H, brs, H-2′), 10.59 (1H, brs, H-1′); HR-ESI-FTMS (*m*/*z*) 762.1757 [(M + H)$^+$, calcd for $C_{37}H_{34}Cl_2N_5O_9$ 762.1734].

Acknowledgment. The authors wish to thank Dr. Z. Guan for high-resolution mass spectral data.

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