New Hydroxyethylamine HIV Protease Inhibitors That Suppress Viral Replication†

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The synthesis of analogues of AcSerLeuAsn[Phe-HEA-Pro]IleValOMe (1, JG-365; where HEA stands for the hydroxyethylamine unit 2), a tight-binding inhibitor of HIVP, are reported. Systematic modification of the P_3 and P_3' regions of the inhibitors has led to smaller HIVP inhibitors that inhibit viral replication in HIV-infected and SIV-infected cell cultures. Six aliphatic and/or aromatic derivatives were prepared by replacing residues in the P_3 regions of BocLeuAsn[Phe-HEA-Pro]IleValOMe. Aromatic side chains at P_3 gave better inhibitors than aliphatic side chains. The better inhibitors in this series contained a β-naphthylalanine or a biphenyl unit at P_3. A second series of HIVP inhibitors were obtained by converting the P_3 group into acyl groups. CbzAsn[Phe-HEA-Pro]IlePheOMe and Qua-Asn-[Phe-HEA-Pro]-Ile-Phe-OMe (where Qua = quinolin-2-ylcarbonyl) are potent HIVP inhibitors with K_i values equal to 1.0 and 0.1 nM, respectively. The inhibition constants were determined by using the continuous fluorometric assay developed by Toth and Marshall. The activities of the protease inhibitors for inhibition of SIV replication were determined in vitro using CEM×174 cells. Inhibition of HIV infection was determined essentially as reported by Pauwels and co-workers. The anti-HIV assay was carried out in culture using CEM cells (a CD4+ lymphocyte line) infected with virus strain $HTLV\text{-}III_b$ with a multiplicity of infection of 0.1. Several analogues inhibited the cytopathic effect at concentrations of 0.1–0.8 μg/mL. These results establish that good inhibitors of HIV protease that inhibit viral replication in infected lymphocytes in in vitro cell assays can be obtained from JG-365 when the AcSerLeu unit is replaced by aromatic acyl derivatives.

Introduction

Human immunodeficiency virus (HIV) is the etiological agent of the disease acquired immune deficiency syndrome (AIDS).[1] Numerous efforts to develop therapeutic agents that inhibit or prevent the development of AIDS have been reported,[2-6] and of the key steps identified to date, proteolytic processing of the polyprotein encoded by the *gag pol* gene of the retrovirus, has attracted much attention. This step, which yields the virally encoded enzymes HIV protease (HIVP), reverse transcriptase, and integrase,[7] is known to be effected by HIVP itself, since mutant systems in which the catalytic sequence is modified are noninfectious.[8] Inhibitors of HIVP are therefore considered particularly promising therapeutic targets.

HIV protease, in common with many other retroviral proteases, is a member of the aspartic proteinase class of

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† This paper is dedicated to Ralph Hirschmann on the occasion of his 70th birthday. This issue of the *Journal of Medicinal Chemistry* honors his scientific leadership and achievements, and tries to indicate his impact on medicinal chemistry and its practitioners. In my case, Ralph Hirschmann supported my efforts to design and synthesize aspartic protease inhibitors based on statine at an early, critical time in my career. Without that support, my contributions to aspartic proteases and to HIV protease inhibitors would not have been made. I am indebted to him for his encouragement, support, and, most of all, for his friendship.

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hydrolytic enzymes.[9-13] The enzyme is dimeric, with each of two identical subunits contributing one AspThrGly catalytic triad to the enzyme active site.[14,15] Thus the active site of HIV protease closely resembles other well-characterized aspartic proteinases such as penicillopepsin,[16] endothiapepsin,[17] rhizopuspepsin,[18] and human renin[19] and is in agreement with earlier structural models.[20]

The discovery that mature HIV protease is an aspartic proteinase suggested to us and others[4,21] that the general design strategy of replacing the P_1-P_1' cleavage point in substrates with transition-state analogues could be used as a basis for the design of tight-binding inhibitors of HIV protease. This strategy[22,23] has provided inhibitors of most aspartic proteinases including pepsin, penicillopepsin, cathepsin D, and renin, with the latter enzyme providing a major impetus into aspartic proteinase inhibitor design.[24]

We therefore chose to synthesize substrate analogues in which a dipeptide transition state analogue replaced the scissile bond. An analogue of the p17/p24 sequence, SerLeuAsnPheProIleVal, was chosen for study because it contained the unusual Phe/TyrPro sequence not normally found in aspartic protease substrate sequences,[25] and a variety of potential reaction pathway mimetics were inserted in place of the PhePro cleavage sequence. These studies led to the discovery of AcSerLeuAsn[Phe-HEA-Pro]IleValOMe (1, where HEA stands for the hydroxyethylamine unit 2), a tight-binding inhibitor of HIVP.[26] The HEA unit 2 was designed on the basis of its resemblance to the tetrahedral intermediate 3 which is produced during the hydrolysis of the PhePro amide bond.

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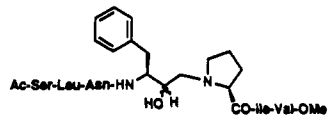
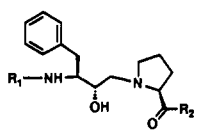


1RS; JG-365



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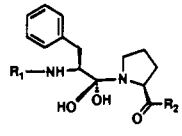

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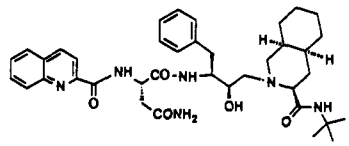

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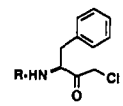
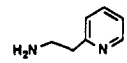


5a, R = Boc-

5b, R = HCl,H



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A related series of HEA inhibitors was simultaneously reported by Roberts et al.,[27] leading to Ro 31-8959 (4), an HIVP inhibitor now in phase II clinical trials for the treatment of AIDS. Interestingly, the preferred configuration of the critical hydroxyl group[28] differs between inhibitors 1 and 4; in 1, maximal inhibition is obtained with the (S)-alcohol diastereomer, whereas the (R)-diastereomer is preferred in 4. These preferences arise from subtle differences in the mode of binding of each inhibitor to the protease.[28-30]

Preliminary evaluation of JG-365 in HIV- and SIV-infected cell cultures indicated that 1 and closely related analogues were not effective inhibitors of HIV replication in cell cultures. Therefore, we carried out synthetic studies on these inhibitors to see if improved inhibitors might be obtained. Systematic modification of the P_3 and P_3' regions of the inhibitors has led to smaller HIVP inhibitors that inhibit viral replication in HIV-infected and SIV-infected cell cultures.

Methods

Synthesis of Inhibitors. The hydroxyethylamine subunits were synthesized as equal mixtures of epimeric (R/S)-alcohols by using the procedure developed by Gordon and co-workers.[31] Phenylalanine chloromethyl ketone (5; Phe-CMK) was synthesized as described and stepwise coupled with BocAsn, BocLeu, and BocSer(Bzl) to give the tetrapeptide chloromethyl ketone 10 (Scheme I). Alkylation of ProIleValOMe (11) with peptidyl CMK 10, followed by reduction with sodium borohydride, gave the HEA peptides as 1:1 mixtures of hydroxyl epimers.[26] The synthesis of 1 is illustrated in Scheme I. The remaining HEA inhibitors shown in Table I were prepared by replacing Leu and Val in intermediates 9 and 11 with the indicated amino acids. The truncated inhibitors shown in Table II were prepared by reaction of BocAsnPhe-CMK (8a) with analogues of tripeptide 11 to give derivatives 25–38. A few diastereomerically pure (S)-HEA diastereomers were prepared by opening the phenylalanine derived (R)-epoxide 39 (Scheme II) with tripeptide 11 or the appropriate tripeptide analogue in the presence of a base, e.g. triethylamine.[28] The (R)-epoxide 39 was prepared via stereoselective epoxidation of allyl amine with m-chloroperbenzoic acid according to the procedure reported by Luly et al.[32a] (Scheme II). The mixture of diastereomers was separated by flash chromatography and the stereochemistry assigned by comparison with the reported NMR data.[32b] Reaction of the (R)-epoxide 39 with tripeptide 11 in refluxing methanol, followed by the usual transformations, gave the (S)-diastereomer of 1. Peptides 29, 37, and 38, which contain the (S)-hydroxyl configuration in [Phe-HEA-Pro], were prepared from 40 (P_3' modified tripeptide analogues of 11) by straightforward modifications of the route shown in Scheme III. Typical procedures for the preparation of 1, 1S, 22 and 39 are given in the Experimental Section. The remaining inhibitors listed in the tables were synthesized in the same manner, but procedures are not given.

HIV Protease Assay. HIV protease activity (enzyme concentration = 6.2 nM) was measured at pH 6.4 with synthetic heptapeptide AntThrIleNlePhe(p-NO_2)Gln-ArgNH_2 by using the continuous fluorometric assay developed by Toth and Marshall.[33] Inhibition constants, assuming pure competitive tight-binding inhibition, were obtained by nonlinear least-squares fit to Morrison's equation for tight binding inhibition[34] by using FastKi, a software program developed for that purpose.[35] K_i values are presented in Tables I and II. The standard error was typically within ±10–30%.

Cell Culture Assays. The activity of protease inhibitors for inhibition of HIV and SIV replication were

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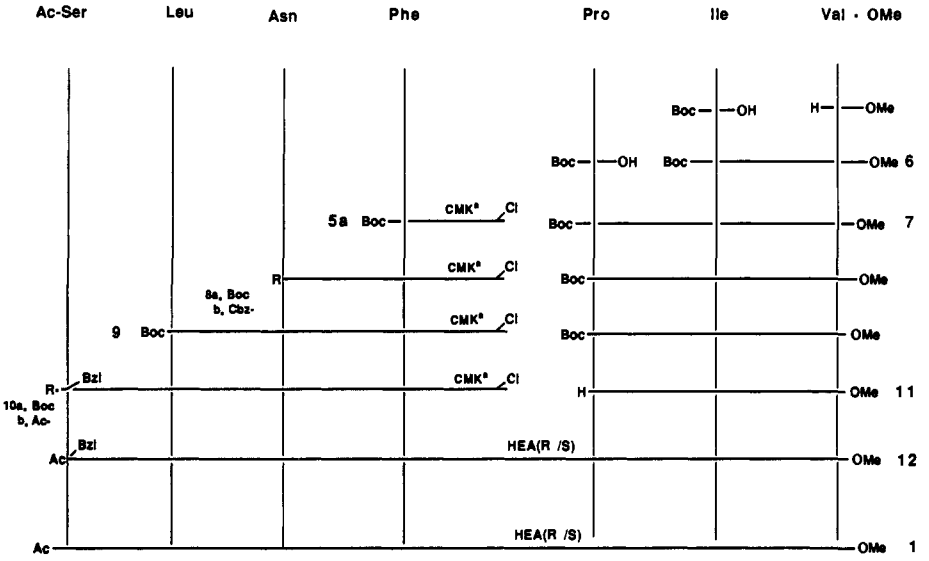
Scheme I. Synthesis of (3*R*/*S*)-Hydroxyethylamine HIV Protease Inhibitors



[a] CMK designates a peptidyl- or amino acid-derived chloromethyl ketone.

Table I. Physical Constants and HIV Protease Inhibition Data for HEA Analogues of JG-365 (1)

no.	structure[e]								K_i,[a] nM	TLC R_f (solv syst)	NMR[b]	HR-FAB MS[c] mol formula
1	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	0.6		+	$C_{49}H_{75}N_9O_{11}$
1S	Ac	Ser	Leu	Asn	[Phe-HEA(*S*)-Pro]	Ile	Val	OMe	0.24[d]	0.06 (A), 0.36 (B)	+	$C_{49}H_{75}N_9O_{11}$
13		Ac	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	4	0.15 (A)	+	$C_{39}H_{64}N_7O_9$
14		Boc	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	66	0.18 (A)	+	$C_{42}H_{70}N_7O_{10}$
15		Boc	HPhe	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	20	0.28 (A)	+	$C_{48}H_{70}N_7O_9$
16		Boc	2-Nal	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	7.8	0.31 (A)	+	$C_{48}H_{70}N_7O_9$
17		Boc	*p*-Bipa	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	14	0.29 (A)	+	$C_{51}H_{72}N_7O_{10}$
18		Boc	*m*-Bipa	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	6.8	0.27 (A)	+	$C_{51}H_{72}N_7O_{10}$
19		Boc	Tyr(I_2)	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	131	0.15 (A)	+	$C_{45}H_{66}N_7O_{11}I_2$
20	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Trp	OMe	3.6	0.05 (A), 0.41 (B)	+	$C_{48}H_{70}N_9O_{11}$
21	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	2-Nal	OMe	5.1	0.08 (A), 0.40 (B)	+	$C_{50}H_{71}N_9O_{11}$
22	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	OMe	0.67	0.09 (A), 0.38 (B)	+	$C_{46}H_{69}N_9O_{11}$
23	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	HPhe	OMe	1.26	0.09 (A), 0.41 (B)	+	$C_{47}H_{71}N_9O_{11}$

[a] For the details of the assay see ref 33. Compound was homogeneous when analyzed by TLC in the indicated solvent: A = 10% MeOH–$CHCl_3$; B = *n*-BuOH–AcOH–H_2O (4:1:1). [b] NMR was consistent with the assigned structure. [c] High-resolution MS was consistent with the empirical formula given. [d] $[\alpha]^{25}_D$ −65.7° (*c* 0.6, MeOH). [e] Abbreviations: HEA, hydroxyethylamine; HPhe, homophenylalanine; 2-Nal, 2-naphthylalanine; *p*-Bipa, *p*-biphenylalanine; *m*-Bipa, *m*-biphenylalanine; Tyr(I_2) diiodotyrosine.

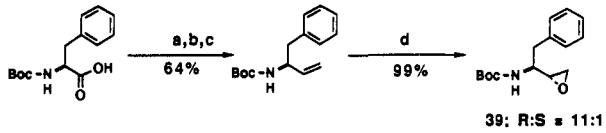
Table II. Physical Constants and HIV Protease Inhibition Data for HEA Analogues of JG-365 (1)

no.	structure[g]						K_i,[a] nM	TLC R_f (solv syst)	$[\alpha]_D$, deg	NMR[b]	HR-FAB MS mol formula[c]
25	Boc	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	440	0.2 (A)		+	$C_{36}H_{58}N_8O_9$
26	Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	9.7	0.22 (A)		+	$C_{39}H_{56}N_8O_9$
27	Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	2.5	0.16 (A)		+	$C_{41}H_{55}N_7O_8$
28	Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	OMe	1.7	0.25 (A), 0.48 (B)		+	$C_{43}H_{57}N_8O_9$
29	Cbz	Asn	[Phe-HEA(*S*)-Pro]	Ile	Phe	OMe	1.0	0.25 (A), 0.48 (B)	−49.6[d]	+	$C_{43}H_{57}N_8O_9$
30	Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Bnp	OMe	1.9	0.27 (A), 0.49 (B)		+	$C_{47}H_{59}N_8O_9$
31	Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	OMe	0.4	0.26 (A), 0.45 (B)		+	$C_{48}H_{58}N_7O_8$
32	Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Bnp	OMe	0.5	0.43 (A), 0.46 (B)		+	$C_{49}H_{58}N_7O_8$
33	Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	NHMe	1.5	0.20 (A)		+	$C_{43}H_{58}N_7O_8$
34	Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	2-EAP		71	0.20 (A)		+	$C_{40}H_{54}N_7O_7$
35	Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	NHMe	0.8	0.22 (A)		+	$C_{45}H_{57}N_8O_7$
36	Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	2-EAP		13	0.18 (A)		+	$C_{42}H_{53}N_8O_8$
37	Qua	Asn	[Phe-HEA(*S*)-Pro]	Ile	Phe	OMe	0.1	0.26 (A), 0.45 (B)	−49.6[e]	+	$C_{45}H_{58}N_7O_8$
38	Cbz	Asn	[Phe-HEA(*S*)-Pro]	Ile	Phe	OH	2.4	0.18 (A), 0.48 (B)	−70.5[f]	+	$C_{42}H_{57}N_8O_8$

[a] For the details of the assay, see ref 33. Compound was homogeneous when analyzed by TLC in the indicated solvents TLC solvents: A = 10% MeOH–$CHCl_3$; B = *n*-BuOH–AcOH–H_2O (4:1:1). [b] NMR was consistent with the assigned structure. [c] High-resolution MS was consistent with the empirical formula given. [d] *c* 0.6, DMSO. [e] *c* 0.4, MeOH. [f] *c* 0.4, DMSO. [g] Abbreviations: HEA, hydroxyethylamine; HPhe, homophenylalanine; Qua, = quinolin-2-ylcarbonyl; 2-EAP, 2-pyridylethylamine, see **24**; OH, alcohol derived from phenylalanine; Bnp, β-naphthylalanine.

determined in vitro. CEM×174 cells[36] were pretreated for 30 min with serial 2-fold dilutions of inhibitor prior to the addition of cell-free preparations of SIV_{mac251}.[37] Fresh inhibitor was added 48 h after the initiation of the culture, and cytopathic effect (CPE) was monitored by the appearance of multinucleated giant cells. The IC_{50} values were determined as the concentration of the compounds causing 50% inhibition of CPE as compared to control

Scheme II [a]



[a] Reaction conditions: (a) EDCI, HOBt, NMM, CH_2Cl_2, *N,O*-dimethylhydroxyamine hydrochloride; (b) Lithium aluminum hydride; ether, 0 °C; (c) Ph_3PCH_3Br, KN(TMS)$_2$; THF, -78 °C; (d) mCPBA, methylene chloride, 0 °C, then separated by silica gel chromatography.

infected cultures after 7-day incubations. The positive control was AZT. Data are presented in Table III.

Inhibition of HIV infection was determined essentially as reported by Pauwles and co-workers.[38] The anti-HIV assay was carried out in culture using CEM cells (a CD4+ lymphocyte line) infected with virus strain HTLV-III$_b$ with a multiplicity of infection (MOI) of 0.1. This moderate MOI insured several rounds of virus multiplication. The endpoint was the virus-induced CPE at day 7 postinoculation, which was quantitated by using the formazan dye technique. Test compounds were added 1 h prior to virus inoculation. In the case of test compounds expected to be metabolically unstable to cell culture, e.g., peptides, additional drug was added on days 2 and 5 postinfection. Controls were untreated cells, virus-infected cells, and test compound-treated cells. The effective concentration for 50% inhibition of the virus-induced CPE was calculated by using linear-regression analysis. Our positive control was AZT with $EC_{50} = 68$ nM ($n = 49$) with 95% confidence intervals of 29–107 nM.

Results

Structure–activity data established that the P_5–P_4 region of JG-365 was not critical for inhibition of the isolated protease; replacement of the AcSer unit by an acetyl group (**1** vs **13**; Table I) weakened inhibition only 6-fold (Table I). Replacement of the acetyl group by the Boc group (**14**) led to significant loss in inhibitory potency (16-fold) and further shortening of the peptide chain led to much weaker inhibitors.[26] Six aliphatic and/or aromatic derivatives (**14**–**19**) were prepared by replacing residues in the P_3 regions of Boc derivative **14** (Table I). The data obtained suggested that aromatic side chains at P_3 were superior to aliphatic side chains. The better inhibitor in this series contained a β-naphthylalanine (**16**) or a biphenyl (**18**) unit at P_3.

In a similar fashion, the structures of the amino acid side chains in the P_3' region of the inhibitor were varied by introducing the amino acids shown in structures **20**–**23** (Table I). In this series of inhibitors, aromatic side chains did not enhance binding to the protease. Inhibitors essentially equipotent to JG-365 were obtained when phenylalanine or homophenylalanine were used in place of valine.

Table II lists a second series of HIVP inhibitors that were obtained by converting the P_3 group in structures **13**–**19** into acyl groups. A comparison of the Boc derivative **25** with the Cbz derivative **26** indicated that aromatic *N*-acyl groups were preferred, as expected from the data in Table I. However, the differences are much greater in this analogue series in that the Cbz derivative is about 44-times more active than the Boc derivative. Replacement of the Cbz-group by a quinolin-2-ylcarbonyl group (Qua), which was introduced into HIVP inhibitors by Roche,[27] increased potency an additional 4-fold (**27**). Within the series of compounds shown in Table II, aromatic substituents at P_3' (e.g. **28**, **30**) significantly enhanced binding to the enzyme relative to valine at P_3' (**26**). The most potent inhibitor is QuaAsn[Phe-HEA-Pro]Ile-PheOMe (**37**). Comparison of analogues **28** and **29** shows that the activity resides in the (*S*)-diastereomer, as expected from the clear preference for the (*S*)-alcohol diastereomers in HIVP inhibitors related to JG-365.[28] Modification of the C-terminal ester group established that amides retained inhibitory potency as HIVP inhibitors (**33**, **35**). Thus, the amide **33** is approximately equipotent with **28**, and **35** is only one-half as active as **31**. The alcohol **38** is about 2–3-fold less active than **29**, but still a good inhibitor of HIVP. In contrast, replacement of the ester group by smaller charged groups (e.g. **24**), produced much weaker inhibitors (**34**, **36**) of the isolated protease.

Some of the more active inhibitors in Table II were evaluated for activity in cell culture. Initially, lymphocytes that were acutely infected with SIV were used to screen for antiviral activity and toxicity. The more promising derivatives were also examined in HIV-infected cell lines. In both assay systems, the cells were infected at a high multiplicity of infection; consequently, the amount of compound needed to inhibit syncytia formation or cytopathic effect was correspondingly high.

Discussion

The X-ray crystal structure of JG-365 (**1**) complexed to synthetic HIVP, first announced by Miller et al.,[30a] provided the first structure of a hydroxyl-containing, tight-binding inhibitor bound in the active site of the protease, and the widespread distribution of the coordinates for this structure prior to formal publication by Swain et al.,[30b] facilitated the design of HIVP inhibitors by many other groups. Many additional X-ray structures have been reported subsequently.[39] In spite of this important role JG-365 played in the elucidation of at least one inhibitor binding mode,[28] it was not at all clear that **1** would be a useful starting point for antiviral agents, because preliminary experiments indicated that **1** was not detectably active against the replicating virus in cell cultures. Other closely related analogues of JG-365 that contained the AcSerLeu moiety also did not inhibit viral replication in cell culture in spite of their strong inhibition of the isolated protease (data not shown). At the time this work was in progress, we did not know if these results were caused by

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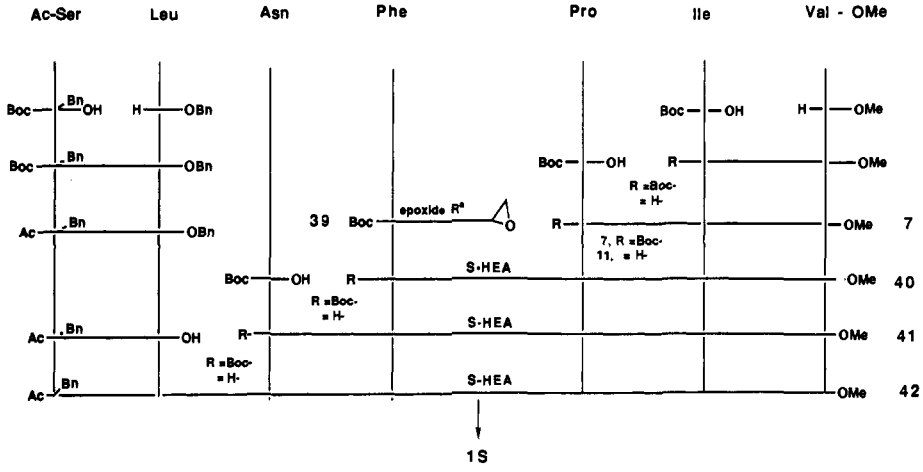
Scheme III. Synthesis of Diastereomerically Pure (*S*)-Hydroxyethylamine (HEA) Inhibitors.



[a] epoxide refers to epoxide 39.

Table III. Inhibition of SIV and HIV Replication by JG-365 (1), 28, and Related Analogues

no.	structure								SIV-induced syncyticia formation: IC_{50},[a] μM	inhibn of HIV-infected CEM,[b] μg/mL
1	Ac	Ser	Leu	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Val	OMe	NA	NA
28			Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	OMe	8	0.60
33			Cbz	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	NHMe	4	0.30
29			Cbz	Asn	[Phe-HEA(*S*)-Pro]	Ile	Phe	OMe	4	0.31
35			Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	Phe	NHMe	4	0.13
36			Qua	Asn	[Phe-HEA(*RS*)-Pro]	Ile	2-EAP		4	0.19
38			Cbz	Asn	[Phe-HEA(*S*)-Pro]	Ile	Phe	OH	T	0.80

[a] For the details of the assay see refs 36 and 37. [b] For the details of the assay see ref 38. NA = not active at highest test concentrations (>10 μM). T = toxic at the assay concentrations. Other inhibitors showed toxicity only at much higher concentrations, generally >100 times the concentrations shown in the table. See footnote *g* of Table II for abbreviations.

enzymatic cleavage of the inhibitor somewhere within the AcSerLeuAsn sequence or due to a failure to enter the cells or some other property of the molecule. Therefore, we decided to modify the structure of JG-365, especially at the N- and C-terminal segments, to see if in vivo activity might be achieved. Some of the inhibitors synthesized for that purpose are shown in Tables I and II.

The crystal structure of JG-365 complexed to HIVP[30] helped guide the design of the synthetic targets. Examination of the crystal structure for potential sites for modification revealed that aromatic groups in the P_3 and P_3' subsites should be accommodated in the enzyme active site and might lead to better inhibitors by virtue of increased hydrophobic interactions between the inhibitor and the enzyme. To test this hypothesis, we synthesized a series of analogues of 14, in which the P_3 and P_3' subsites were systematically varied. Peptide 14 was chosen as a model system for this study, because 14 was relatively easy to synthesize and of intermediate potency so that substitutions that gave significant increases in activity would be easy to detect. As shown in Table I, aromatic substituents at P_3 do increase binding, as suggested from the X-ray crystal structure. However, the effect of aromatics at P_3' was evident only in the shorter inhibitors (Table II). In an attempt to obtain lower molecular weight derivatives that might be active in cell culture, we further shortened the inhibitors. The P_5-P_3 unit could be replaced with an aromatic acyl group, e.g. Cbz (**26**), to give analogues with good activity. Comparison of **25** vs **26** indicated that a benzyloxycarbonyl group was superior to a *tert*-butyloxycarbonyl group in this series of analogues. Later, following the lead reported by the Roche group,[27] we placed the Qua group at this position to obtain derivatives with good activity in cell cultures, e.g. **27**. When aromatic substitutions were introduced into both the P_3 and P_3' subsites of **25**, excellent inhibitors of HIVP (**28–32**) were obtained. Here, the beneficial effect on binding of aromatic substituents at P_3' anticipated from the X-ray crystal structure of the enzyme-JG-365 complex is clearly evident. Further reduction in peptide chain length, e.g. those formed by deleting the Val and IleVal units, gave much weaker inhibitors as previously reported.[26]

The inhibitors in Table II retain other potential sites for metabolic inactivation in the C-terminal region. Most notably, inhibitors **26–32** contain a methyl ester, which could undergo hydrolysis in vivo. In order to determine if the ester group is essential for inhibitory activity, we introduced more stable units in the C-terminal segment. The *N*-methylamide analogues **33** and **35** and the alcohol **38** retain good activity. Thus, an intact C-terminal ester group is not a requirement for activity in this series of inhibitors.

The analogues of JG-365 described here are moderately soluble in water due to the presence of the tertiary amine within the HEA unit. Kempf et al. have reported that increased activity in cell culture is associated with increased water solubility.[40] Compounds **34** and **36** were synthesized in one attempt to see if the same effect might be obtained.

(40) Kempf, D. J.; Marsh, K. C.; Paul, D. A.; Knigge, M. F.; Norbeck, D. W.; Kohlbrenner, W. E.; Codacovi, L.; Vasavanonda, S.; Bryant, P.; Wang, X. C.; Wideburg, N. E.; Clement, J. J.; Plattner, J. J.; Erickson, J. Antiviral and Pharmacokinetic Properties of C_2 Symmetric Inhibitors of the Human Immunodeficiency Virus Type 1 Protease. *Antimicrob. Agents Chemother.* **1991**, *35*, 2209–2214.

Both analogues are considerably weaker inhibitors of the isolated protease; however, both retain activity against the virus in cell cultures. On a molar basis, **36** is about one-half as active in cells relative to **35** but only $^1/_{16}$ as active as **35** in the isolated protease assay. Thus, the compound is much more effective in in vitro cell culture assay than anticipated from its inhibition of the isolated protease. Whether this is caused by greater metabolic stability or enhanced cell penetration is not known. Further work will be needed to obtain water-soluble, highly potent HIVP inhibitors.

Our results establish that good inhibitors of HIV protease can be obtained from JG-365 when the AcSerLeu unit is replaced by aromatic acyl derivatives. Most importantly, analogues that inhibit viral replication in infected lymphocytes in in vitro cell assays have been obtained. In a long-term culture of persistently HIV-infected HCEM cells,[41] the production of HIV *gag* proteins (p24) in cell culture was approximately 20 times lower (about 400 ng/mL in the absence and about 20 ng/mL in the presence of **28**) after a 72-h exposure to the inhibitor. Inhibitors **35** and **36** had similar effects.[42] Details of these studies will be reported separately.[42]

Experimental Section

Melting points were determined with Fisher-Johns melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were recorded on either Bruker WP200 (200 MHz) or Bruker AM-300 (300 MHz) Fourier transform spectrometers. Chemical shifts were reported as δ units (parts per million) relative to tetramethylsilane as internal standard (δ = 0.00). Optical rotations were determined on a Perkin-Elmer Model 241 automatic polarimeter (0.9999-dm cell). TLC was performed on 0.25-mm thickness silica gel plates (Merck, silica gel 60 F-254). For column chromatography, Merck grade 60 silica gel, 230–400 mesh, was used. Compounds were visualized on the plates by reaction with ninhydrin, 5% phosphomolybdic acid in ethanol, and ultraviolet light. All compounds used in protease assay appeared as single spot on TLC. IC_{50}s were measured with synthetic AntThrIleNlePhe(*p*-NO_2)GlnArgNH_2 as substrate, by fluorometric assay as described in the literature.[33]

General Procedures: A. Isobutyl Chloroformate-Mediated Coupling Reactions. To a cold (−25 °C) solution of N_α-protected amino acid (100 mmol) in dry dichloromethane (200–400 mL) is added *N*-methylmorpholine (100 mmol) followed by careful addition of isobutyl chloroformate (100 mmol) while the temperature of the solution is maintained between −15 and −25 °C. The solution is stirred at −25 °C for a further 20 min, and then a solution of amino acid or peptide ester hydrohalide or toluenesulfonate salt (100 mmol) in DMF and/or DCM is added followed by addition of NMM (100 mmol). The reaction is stirred for 1 h at −20 °C and for a further 1–3 h at room temperature. The solvent is removed in vacuo and the residue taken up in ethyl acetate and washed with saturated $NaHCO_3$ (×2), 5% citric acid solution, saturated $NaHCO_3$, water, and brine and dried over $MgSO_4$. Evaporation of the solvent yields a solid which is recrystallized from a suitable solvent combination.

B. EDCI/HOBt-Mediated Peptide Couplings. Boc amino acid and HOBt (1.5 equiv) are dissolved in dry DMF (2–5 mL/mmol) and cooled to 0 °C. EDCI (1.1 equiv) is added and the solution stirred for 30–40 min. A solution of amino acid or peptide (ester, chloromethyl ketone, or amine hydrochloride) (0.9 equiv) in DMF (1–5 mL/mmol) is added, followed by NMM (0.9 equiv). The reaction is stirred for 1–2 h at 0 °C and overnight at room temperature. The reaction is worked up by direct precipitation of the crude product from a concentrated DMF solution by careful addition of a large volume of 60% saturated $NaHCO_3$. The precipitate is collected by filtration over hardened filter paper (Whatman No. 50 or 54), and the solid washed thoroughly with saturated $NaHCO_3$ (200–500 mL), water (200–500 mL), 5% citric acid solution (200–500 mL), and water (200–500 mL). The product is dried in vacuo, redissolved in DMF, filtered through a glass-wool plug, and reprecipitated by addition of water. Again the product is filtered, washed with water, and dried.

C. Cleavage of Boc Protecting Group with 4 N HCl in Dioxane. The Boc-protected peptide or amino acid derivative is dissolved in 4 N HCl in dioxane (Pierce) (6–40 equiv) and stirred at room temperature for 30–60 min, the course of the reaction being monitored by TLC. Once complete, the solution is evaporated to dryness at <30 °C and the residue evaporated from anhydrous ether (×3) to remove traces of HCl. The crude product is dried in vacuo in a desiccator over NaOH pellets overnight and is used without further purification or characterization.

D. Preparation of Amino Acid Diazomethyl Ketones. A typical procedure is as follows: N_α-protected amino acid (50 mmol) is dissolved in dry THF (100 mL) and cooled to −25 °C. NMM (50 mmol) is added, followed by isobutyl chloroformate (50 mmol), with the temperature being maintained at −25 °C during addition. After stirring for 5 min, anhydrous ether (100 mL) is added while the solution is simultaneously cooled to −70 °C. The cold solution is quickly filtered under a blanket of N_2 and the cold filtrate treated with a solution containing approximately 70 mmol of diazomethane in ether (250 mL). The reaction is allowed to gradually warm up to room temperature and excess diazomethane is removed by purging the solution with a stream of N_2 for 15–30 min. The solution is evaporated to dryness and the residue taken up in ether (200 mL), washed with saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. The product may be crystallized from ether–hexane or hexane.

E. Conversion of Diazomethyl Ketones to Chloromethyl Ketones. The amino acid diazomethyl ketone is dissolved in anhydrous dioxane or ether (10 mL/g), cooled to 0 °C, and treated with a solution of 4 N HCl in dioxane. After slow addition of the first equivalent of HCl in dioxane is complete (slow addition is necessitated by the rapid evolution of nitrogen), a further 6–10 equiv is added and the cooling bath removed. The reaction is stirred at room temperature for 1 h and the product precipitated by addition of ether. The product is filtered, washed with copious amounts of ether, and dried in vacuo over NaOH pellets. Prolonged storage of these compounds results in significant coloration and decomposition.

F. Acetylation of Peptides. A solution of the peptide amine hydrochloride in dry DMF (10–20 mL/g) is cooled to 0 °C in an ice–water bath and neutralized with triethylamine (2.1–2.5 equiv). A solution of acetyl chloride in DCM (0.3–1.8 M) is then added dropwise and the reaction left to stir for 1 h at 0 °C and for a further 1 h at room temperature. The solution is then concentrated to a small volume under high vacuum, and water is added to precipitate the acetylated peptide, which is collected by filtration and washed with plenty of water, before drying in vacuo over NaOH pellets.

G. Preparation of Ketomethylenamines. N-Protected peptide chloromethyl ketone derivatives (1.0 mmol) are dissolved in DMF (4–7 mL/mmol), and NaI (1.1 mmol) is added, and the mixture is stirred for 15 min. The peptide ester toluenesulfonate salt (1.1 mmol) in DMF (2–5 mL/mmol) is added followed by $NaHCO_3$ (2.1 mmol) and the reaction left to stir for 12–18 h at room temperature. Upon complete reaction, the solution is diluted with ethyl acetate (50–70 mL) and washed with water (1 N $KHSO_4$ may also be used, though the potential solubility of an amine-containing peptide must be considered). The aqueous layer is extracted with ethyl acetate (×2) and the combined extracts washed with water and brine before drying over Na_2SO_4. The product thus obtained is of good purity, though silica gel chromatography carried out to remove trace impurities contributes to epimerization at the chiral center α to the ketone carbonyl.

H. Reduction of Ketomethylenamines to Hydroxyethylamines. The crude ketomethylenamine (1.0 mmol) is taken

(41) Pauza, D. D.; Galindo, J. Persistent human immunodeficiency virus type 1 infection of monoblastoid cells leads to accumulation of self-integrated viral DNA and to production of defective virions. *J. Virol.* 1989, *63*, 3700–3707.

(42) MacKenzie, D.; Rich, D. H.; Sun, C.-Q.; Pauza, C. D.; Mueller, R. A.; Houseman, K.; Malkovsky, M. Suppression of Viral Replication by Hydroxyethylamine Dipeptide Isostere Inhibitors of HIV Protease, submitted for publication.

up in anhydrous MeOH (10 mL), cooled to 0 °C, and treated with excess $NaBH_4$ in a single portion. The course of reaction is monitored by TLC, and addition of extra $NaBH_4$ is occasionally necessary. Upon completion of reduction, the solution is treated with ethyl acetate (50 mL) and water (50 mL). The aqueous phase is extracted with ethyl acetate (×2), and the combined extracts are washed with water and brine and dried over Na_2SO_4. The product is purified by silica gel chromatography using a gradient of methanol in chloroform for optimal separations. Diastereomer separation is not usually possible.

I. Hydrogenolytic Deprotection of Final Products. The amino alcohol containing peptides (50 μmol) are dissolved in 90% acetic acid (3.5 mL) and 10% palladium hydroxide on charcoal (Pearlman's catalyst) is added under an N_2 atmosphere. Deprotection is achieved by passage of H_2 as a steady stream through the solution for 3–4 h. The catalyst is removed by filtration through prewashed and swollen Celite, and the filtrate concentrated in vacuo to approximately 1 mL total volume. The solution is diluted with water (20 mL), washed with chloroform (×3), and reconcentrated to 5–8 mL total volume. The solution is filtered through a glass-wool plug and lyophilized to yield the final product peptide as a fine white powder.

J. Epoxide Openings with Peptide or Amino Acid Amines. To a stirred solution of an epoxide (1.0 mmol) in dry methanol (10 mL) are added an N-deprotected peptide or amino acid ester hydrochloride (1.2 mmol) and triethylamine (1.2 mmol). The resultant solution is refluxed for 24 h and then evaporated to give a residue, which is purified by silica gel chromatography using 3–4% methanol in chloroform to give the desired product.

Synthesis of JG-365 (1). (3*S*)-[(*tert*-Butoxycarbonyl)amino]-1-diazo-4-phenyl-2-butanone (BocPheCHN$_2$). The title compound was prepared according to general procedure D from Boc-phenylalanine (13.28 g, 50.0 mmol). The product was crystallized from ether–hexane and used immediately in the next step: yield, 13.44 g, 93%; TLC R_f 0.50 (1:1 EtOAc–hexane); mp 96–97 °C; $[\alpha]^{24}_D$ +13.50° (*c* 1.10, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3450, 2990, 2140 (N=N), 1715, 1645, 1500, 1390, 1380, 1250, 1170 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.35–7.17 (5 H, m, aromatic), 5.23 (1 H, s, CHN_2), 5.10 (1 H, br d, NH), 4.43 (1 H, m, C3-H), 3.03 (2 H, d, *J* = 6.8 Hz, C4-H_2), 1.41 (9 H, s, Boc CH_3's).

(3*S*)-Amino-1-chloro-4-phenyl-2-butanone Hydrochloride (5b). The title compound was prepared from (3*S*)-[(*tert*-butoxycarbonyl)amino]-1-diazo-4-phenyl-2-butanone (BocPheCHN$_2$) (10.01 g, 34.6 mmol) in dioxane (25 mL), by reaction with a solution of 4 N HCl in dioxane (50 mL, 200 mmol) as described in general procedure E: yield, 7.86 g, 97%; mp 170 °C dec [lit.[43] mp 169 °C); $[\alpha]^{22}_D$ +33.6° (*c* 1.6, 0.1 N HCl) [lit.[43] $[\alpha]^{20}_D$ +30.2° (*c* 2, 0.1 N HCl)]; ^{1}H NMR (200 MHz, DMSO-d_6) δ = 8.63 (3 H, br, NH_3^+), 7.32 (5 H, m, aromatic), 4.55 (2 H, AB, *J* = 17.5, $COCH_2Cl$), 4.53 (1 H, m, C3-H), 3.17 (2 H, 2 d, *J* = 4.1, 6.6 Hz, C4-H_2).

(3*S*)-[[*N*-(*tert*-Butoxycarbonyl)asparaginyl]amino]-1-chloro-4-phenyl-2-butanone (BocAsnPheCH$_2$Cl) (8a). The title compound was prepared from (3*S*)-amino-1-chloro-4-phenyl-2-butanone hydrochloride (**5b**; 7.04 g, 30.1 mmol) and Boc-asparagine (7.66 g, 33.0 mmol) in DMF (100 mL), as described in general procedure B: yield, 11.59 g, 94%; TLC R_f 0.31 (9:1 $CHCl_3$–MeOH); mp 182–183 °C; $[\alpha]_D$ −100.8° (*c* 0.66, DMSO); ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.46 (1 H, d, *J* = 7.4 Hz, NH), 7.22 (6 H, m, aromatic, Asn $CONH_2$), 6.95 (2 H, m, Asn NH, $CONH_2$), 4.56 (2 H, AB, *J* = 17.1 Hz, $COCH_2Cl$), 4.42 (1 H, m, C3-H), 4.16 (1 H, m, Asn α-CH), 3.25–2.70 (2 H, m, C4-H_2), 2.31 (2 H, d, *J* = 6.7 Hz, Asn β-CH_2), 1.37 (9 H, s, Boc CH_3's).

(3*S*)-[[*N*-(*tert*-Butoxycarbonyl)leucinylasparaginyl]amino]-1-chloro-4-phenyl-2-butanone (BocLeuAsnPheCH$_2$Cl) (9). The title compound was prepared by deprotection of 8 (9.74 g, 23.7 mmol) according to general procedure C, followed by coupling with Boc-leucine (6.55 g, 26.3 mmol) [monohydrate dried together with hydroxybenzotriazole hydrate by repeated azeotropic removal of water from DMF–toluene solution] in DMF (70 mL), as described in general procedure B: yield 8.17 g, 66%; TLC R_f 0.31 (9:1 $CHCl_3$–MeOH); mp 182–185 °C; $[\alpha]_D$ −75.2° (*c* 1.34, DMF); ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.40 (1 H, d, *J* = 7.5 Hz, NH), 8.02 (1 H, d, *J* = 7.8 Hz, Asn NH), 7.37, 6.96 (2 × 1 H, 2 s, Asn $CONH_2$), 7.23 (5 H, m, aromatic), 6.92 (1 H, d, *J* = 7.9 Hz, Leu NH), 4.56 (2 H, AB, *J* = 16.8 Hz, $COCH_2Cl$), 4.47 (2 H, m, C3-H, Leu α-CH), 3.92 (1 H, m, Asn α-CH), 3.30–2.70 (2 H, m, C4-H_2), 2.45 (2 H, m, Asn β-CH_2), 1.80–1.50 (3 H, m, Leu β-CH_2, γ-CH), 1.37 (9 H, s, Boc CH_3's), 0.85 (6 H, 2 d, *J* = 6.4 Hz, Leu δ-CH_3's); FAB MS *m/z* 527, 525, 471, 469, 427, 425, 225; HRMS $C_{25}H_{38}N_4O_6{}^{35}Cl$ requires 525.2480, found 525.2477 (dev −0.5 ppm).

(3*S*)-[[*N*-(*tert*-Butoxycarbonyl)-*O*-benzylserinylleucinylasparaginyl]amino]-1-chloro-4-phenyl-2-butanone (BocSer(Bzl)LeuAsnPheCH$_2$Cl) (10a). The title compound was prepared by deprotection of BocLeuAsnPheCH$_2$Cl (**9**) according to general procedure C, followed by coupling with Boc-serine benzyl ether (0.99 g, 1.89 mmol) in DMF (11 mL), as described in general procedure B: yield, 1.17 g, 88%; TLC R_f 0.48 (9:1 $CHCl_3$–MeOH); mp 175–179 °C; $[\alpha]_D$ −52.7° (*c* 0.98, DMF); ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.42 (1 H, d, *J* = 6.9 Hz, NH), 8.22 (1 H, d, *J* = 7.0 Hz, Asn NH), 7.87 (1 H, d, *J* = 7.7 Hz, Leu NH), 7.40–7.05 (11 H, m, aromatic, Asn $CONH_2$), 7.03 (1 H, d, *J* = 8.5 Hz, Ser NH), 6.96 (1 H, s, Asn $CONH_2$), 4.56 (2 H, AB, *J* = 17.0 Hz, $COCH_2Cl$), 4.55–4.20 (6 H, m, Ser α-CH, Asn α-CH, Leu α-CH, C3-H, Bzl CH_2), 3.59 (2 H, Ser β-CH_2), 3.16–2.80 (2 H, m, C4-H_2), 2.60–2.30 (2 H, obscured by solvent, Asn β-CH_2), 1.59 (1 H, m, Leu β-CH_2), 1.38 (11 H, s, Boc CH_3's, Leu β-CH_2, ν-CH), 0.81 (6 H, 2 d, *J* = 4.8 Hz, Leu δ-CH_3's); FAB MS *m/z* 702, 668, 602, 568, 263; HRMS $C_{35}H_{49}N_5O_8{}^{35}Cl$ requires 702.3270, found 702.3260 (dev −1.0 ppm).

(3*S*)-[(*N*-Acetyl-*O*-benzylserinylleucinylasparaginyl)amino]-1-chloro-4-phenyl-2-butanone (AcSer(Bzl)LeuAsnPheCH$_2$Cl) (10b). The title compound was prepared from BocSer(Bzl)LeuAsnPheCH$_2$Cl (**10a**; 674 mg, 0.96 mmol), by deprotection according to general procedure C and acetylation according to general procedure F: yield, 582 mg, 94%; TLC R_f 0.19 (9:1 $CHCl_3$–MeOH), 0.39 (9:1 CH_2Cl_2–MeOH), 0.46 (85:10:5 $CHCl_3$–MeOH–AcOH), 0.74 (3:1:1 *n*-BuOH–AcOH–H_2O); mp 191–194 °C dec; $[\alpha]_D$ −58.7° (*c* 0.53, DMF); ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.36 (1 H, d, *J* = 7.5 Hz, NH), 8.16 (1 H, d, *J* = 7.6 Hz, Asn NH), 8.09 (1 H, d, *J* = 7.4 Hz, Ser NH), 7.95 (1 H, d, *J* = 7.7 Hz, Leu NH), 7.35–7.17 (11 H, m, aromatic, Asn $CONH_2$), 6.95 (1 H, s, Asn $CONH_2$), 4.55 (2 H, AB, *J* = 17.1 Hz, $COCH_2Cl$), 4.69–4.25 (8 H, m, $COCH_2Cl$, Ser α-CH, Asn α-CH, Leu α-CH, C3-H, Bzl CH_2), 3.59 (2 H, d, *J* = 5.5 Hz, Ser β-CH_2), 3.16–2.80 (2 H, m, C4-H_2), 2.55–2.25 (2 H, obscured by solvent, Asn β-CH_2), 1.88 (3 H, s, Ac CH_3), 1.70–1.30 (3 H, m, Leu β-CH_2, γ-CH), 0.83 (6 H, m, Leu δ-CH_3's); FAB MS *m/z* 644, 610, 447, 225; HRMS $C_{32}H_{43}N_5O_7{}^{35}Cl$ (MH^+) requires 644.2851, found 644.2841 (dev −1.5 ppm).

(2*RS*,3*S*)-3-[(*N*-Acetyl-*O*-benzylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester)butane (AcSer(Bzl)LeuAsn[Phe-HEA(*RS*)-Pro]IleVal-OMe) (12). The title compound was prepared from AcSer(Bzl)LeuAsnPheCH$_2$Cl (188 mg, 0.292 mmol) and prolylisoleucylvaline methyl ester toluenesulfonate (**11**; 223 mg, 0.435 mmol) in DMF (4.5 mL) as described in general procedure G. The crude product was reduced without further purification using $NaBH_4$ (28 mg, 0.733 mmol) as described in general procedure H. The crude product from aqueous workup was dried by azeotropic removal of water to prevent precipitation and purified by chromatography on silica gel, using a gradient of 5–11% MeOH in $CHCl_3$: yield, 129 mg, 46%; TLC R_f 0.29 (9:1 $CHCl_3$–MeOH), 0.40 (9:1 CH_2Cl_2–MeOH), 0.19, 0.11 (85:10:5 $CHCl_3$–MeOH–AcOH); FAB MS *m/z* 951, 679, 486, 354, 259; HRMS $C_{49}H_{75}N_8O_{11}$ (MH^+) requires 951.5555, found 951.5556 (dev 0.0 ppm).

(2*RS*,3*S*)-3-[(*N*-Acetylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester)butane (AcSerLeuAsn[Phe-HEA(*RS*)-Pro]Ile-ValOMe·AcOH) (1). The title compound was prepared from

(43) Fittkau, V. S. α-Aminochloromethylketone aus Aminosauren und peptiden als substratanaloge Inhibitore der Leucinaminopeptidase. *J. Prakt. Chem.* **1973**, *315*, 1037–1041.

(44) Abbreviations used follow IUPAC–IUB tentative rules as described in *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used are as follows: Ant, 2-aminobenzoyl (from anthranilic acid); DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; Boc, *tert*-butyloxycarbonyl; NMM, *N*-methylmorpholine; DMF, dimethylformamide; THF, tetrahydrofuran; 2-Nal; 2-naphthylalanine; Qua, quinolin-2-ylcarbonyl.

AcSer(Bzl)LeuAsn[Phe-HEA-Pro]IleValOMe (12; 36 mg, 0.0378 mmol) by hydrogenation over palladium hydroxide on charcoal (14 mg) in 90% acetic acid as described in general procedure I: yield, 34 mg, 99% (hydrated); FAB MS *m/z* 899 (MK^+), 883 (MNa^+), 867 (MLi^+), 861 (MH^+), 589, 259; HRMS $C_{42}H_{69}N_8O_{11}$ (MH^+) requires 861.5086, found 861.5078 (dev −0.9 ppm).

Synthesis of (3*S*)-Hydroxy Derivatives. (2*S*,3*S*)-3-[*N*-(*tert*-Butoxycarbonyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester)butane (Boc[Phe-HEA-Pro]IleValOMe) (40). The title compound was prepared (0.58 g, 87% yield) from (1*R*)-[1′(*S*)-[*N*-(*tert*-butoxycarbonyl)amino]-2′-phenylethyl]oxirane (**39**;[32] 0.29 g, 1.10 mmol) and prolylisoleucylvaline methyl ester hydrochloride (11; 0.50 g, 1.34 mmol) according to general procedure J: TLC R_f 0.71 (10% MeOH–$CHCl_3$), 0.26 (1:1 EtOAc–hexane); 0.54 (4:1:1 *n*-BuOH–AcOH–H_2O); $[\alpha]^{25}_D$ −87.1° (*c* 1.0, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88–1.00 (m, 12 H), 1.10–1.60 (m, 3 H), 1.37 (s, 9 H), 1.70–1.90 (m, 4 H), 2.05–2.30 (m, 3 H), 2.35–2.75 (m, 2 H), 2.92 (m, 2 H), 3.07–3.22 (m, 2 H), 3.62–3.74 (m, 1 H), 3.76 (s, 3 H), 4.23–4.40 (m, 2 H), 4.60 (m, 1 H), 5.10 (d, *J* = 9.8 Hz, 1 H), 6.52 (d, *J* = 9.5 Hz, 1 H), 7.18–7.30 (m, 5 H), 8.21 (d, *J* = 9.5 Hz, 1 H); exact mass calcd for $C_{32}H_{53}N_4O_7$ $(M + H)^+$ 605.3914, found (HR-FAB MS) 605.3914.

(2*S*,3*S*)-3-[[*N*-(*tert*-Butoxycarbonyl)asparaginyl]amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester)butane (BocAsn[Phe-HEA-Pro]IleValOMe) (41). The title compound was prepared (0.33 g, 53% yield) by two-step reactions, first N-deprotection of **40** (0.53 g, 0.86 mmol) according to general procedure C, followed by coupling with Boc-AsnOH (0.232 g, 1.00 mmol) according to general procedure B. The product was purified by flash chromatography using 4–6% methanol in chloroform: TLC R_f 0.35 (10% MeOH–$CHCl_3$), 0.42 (4:1:1 *n*-BuOH–AcOH–H_2O); $[\alpha]^{25}_D$ −77.5° (*c* 0.4, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88–1.02 (m, 12 H), 1.05–2.00 (m, 5 H), 1.43 (s, 9 H), 2.05–3.05 (m, 9 H), 3.10–3.20 (m, 2 H), 3.72 (m, 1 H), 3.76 (s, 3 H), 3.96 (q, *J* = 7.9 Hz, 1 H), 4.28 (dd, *J* = 6.0, 8.5 Hz, 1 H), 4.32–4.45 (m, 2 H), 4.59 (dd, *J* = 5.3, 8.5 Hz, 1 H), 5.66 (bs, 1 H), 5.86 (d, *J* = 8.5 Hz, 1 H), 6.03 (bs, 1 H), 6.52 (d, *J* = 8.5 Hz, 1 H), 7.15–7.28 (m, 5 H), 8.08 (d, *J* = 8.5 Hz, 1 H); exact mass calcd for $C_{36}H_{59}N_6O_9$ $(M + H)^+$ 719.4344, found (HR-FAB MS) 719.4335.

(2*S*,3*S*)-3-[(*N*-Acetyl-*O*-benzylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester)butane (AcSer(Bn)LeuAsn[Phe-HEA(*S*)-Pro]IleValOMe) (42). The title compound was prepared (160 mg, 44% yield) by two-step reactions, first N-deprotection of **41** (290 mg, 0.403 mmol) according to general procedure C, followed by coupling with AcSer(Bn)LeuOH (155 mg, 0.443 mmol) according to general procedure B. The product was purified by flash chromatography using a gradient of 4, 6, 8% methanol in chloroform: TLC R_f 0.24 (10% MeOH–$CHCl_3$), 0.42 (4:1:1 *n*-BuOH–AcOH–H_2O); $[\alpha]^{25}_D$ −67.8° (*c* 0.45, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.78–0.93 (m, 18 H), 0.95–1.10 (m, 2 H), 1.20–2.20 (m, 9 H), 1.89 (s, 3 H), 2.25–2.70 (m, 5 H), 2.81–2.90 (m, 1 H), 3.00–3.10 (m, 2 H), 3.60 (s, 3 H), 3.59–3.62 (m, 3 H), 3.90 (m, 2 H), 4.18 (t, *J* = 7.0 Hz, 1 H), 4.00–4.55 (m, 6 H), 5.10 (d, *J* = 5.4 Hz, 1 H), 6.85 (bs, 1 H), 7.10–7.39 (m, 12 H), 8.00 (m, 2 H), 8.19 (m, 2 H); exact mass calcd for $C_{49}H_{75}N_8O_{11}$ $(M + H)^+$ 951.5555, found (HR-FAB MS) 951.5559.

(2*S*,3*S*)-3-[(*N*-Acetylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucylvaline methyl ester(butane (AcSerLeuAsn[Phe-HEA(*S*)-Pro]IleValOMe·AcOH) (1S). The title compound was prepared (105 mg, 99% yield) from **42** (110 mg, 0.115 mmol) according to general procedure I: TLC R_f 0.06 (10% MeOH–$CHCl_3$), 0.36 (4:1:1 *n*-BuOH–AcOH–H_2O); $[\alpha]^{25}_D$ −65.7° (*c* 0.6, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.75–0.93 (m, 18 H), 1.05 (m, 1 H), 1.35–2.15 (m, 13 H), 1.88 (s, 3 H), 2.20–2.65 (m, 5 H), 2.88 (m, 1 H), 3.07 (m, 2 H), 3.61 (s, 3 H), 3.40–4.00 (m, 3 H), 4.10–4.50 (m, 7 H), 6.87 (bs, 1 H), 7.10–7.38 (m, 7 H), 7.80–8.30 (m, 5 H); exact mass calcd for $C_{42}H_{69}N_8O_{11}$ $(M + H)^+$ 861.5086, found (HR-FAB MS) 861.5094.

P-3′ Modifications of (*R*/*S*)-JG-365 (1). *N*-(*tert*-Butoxycarbonyl)isoleucinylphenylalanine Methyl Ester (BocIlePheOMe). The title compound (4.17 g, 92% yield) was prepared from Boc-isoleucine hemihydrate (2.78 g, 11.59 mmol) and phenylalanine methyl ester hydrochloride (2.50 g, 11.59 mmol) according to general procedure B: TLC R_f 0.58 (50% EtOAc–hexane), 0.75 (10% MeOH–$CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90 (m, 6 H), 1.00–1.60 (m, 2 H), 1.45 (s, 9 H), 1.85 (m, 1 H), 3.10 (m, 2 H), 3.73 (s, 3 H), 3.93 (t, *J* = 7.0 Hz, 1 H), 4.90 (q, *J* = 7.0 Hz, 1 H), 5.00 (bs, 1 H), 6.25 (bs, 1 H), 7.05–7.30 (m, 5 H).

***N*-(*tert*-Butoxycarbonyl)prolylisoleucinylphenylalanine Methyl Ester (BocProIlePheOMe).** The title compound (4.38 g, 84% yield) was prepared by a two-step procedure. *N*-Deprotection of BocIlePheOMe (4.17 g, 10.64 mmol) according to general procedure C was followed by coupling with Boc-proline (2.30 g, 10.64 mmol) according to general procedure B. The product was purified by flash chromatography using 50% ethyl acetate in hexane: TLC R_f 0.21 (50% EtOAc–hexane), 0.59 (10% MeOH–$CHCl_3$); mp 99–101 °C; $[\alpha]^{25}_D$ −71.54° (*c* 1.1, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (m, 6 H), 1.05 (m, 1 H), 1.33 (s, 9 H), 1.35–2.20 (m, 6 H), 3.00 (m, 2 H), 3.26 (m, 2 H), 3.56 (s, 3 H), 4.17 (m, 2 H), 4.48 (m, 1 H), 7.20 (m, 5 H), 7.64 (d, *J* = 8.0 Hz, 1 H), 8.42 (d, *J* = 8.0 Hz, 1 H).

(2*R*/*S*,3*S*)-3-[(*N*-Acetyl-*O*-benzylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalanine methyl ester)butane (AcSer(Bn)LeuAsn[Phe-HEA-Pro]IlePheOMe). The title compound (54.7 mg, 20% yield) was prepared from BocProIlePheOMe (159 mg, 0.324 mmol) by a two-step procedure. N-Deprotection according to general procedure C, was followed by reaction with AcSer(Bn)LeuAsnPheCH$_2$Cl (**10b**) (175 mg, 0.27 mmol) according to general procedures G and H: TLC R_f 0.25 (10% MeOH–$CHCl_3$), 0.61 (15% MeOH–$CHCl_3$), 0.32 (4:1:1 *n*-BuOH–AcOH–H_2O); ^{1}H NMR (DMSO-d_6) δ 0.70–0.88 (m, 12 H), 0.95 (m, 1 H), 1.10–1.80 (m, 8 H), 1.90 (s, 3 H), 2.05 (m, 1 H), 2.20–3.20 (m, 11 H), 3.52, 3.55 (2 s, 3 H), 3.60 (d, *J* = 4.0 Hz, 2 H), 3.90 (m, 1 H), 4.10–4.60 (m, 7 H), 5.30 (m, 2 H), 6.90 (m, 1 H), 7.05–7.35 (m, 16 H), 7.40–8.65 (m, 6 H).

(2*R*/*S*,3*S*)-3-[(*N*-Acetylserinylleucinylasparaginyl)amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalanine methyl ester(butane (AcSerLeuAsn[Phe-HEA-Pro]IlePheOMe·AcOH) (22). The title compound (40 mg, 100% yield) was prepared from AcSer(Bn)LeuAsn[Phe-HEA-Pro]IlePheOMe (40 mg, 0.04 mmol) according to general procedure I: TLC R_f 0.09 (10% MeOH–$CHCl_3$), 0.38 (4:1:1 *n*-BuOH–AcOH–H_2O); ^{1}H NMR (DMSO-d_6) δ 0.65–1.00 (m, 12 H), 1.02 (m, 1 H), 1.15–2.20 (m, 11 H), 1.90 (s, 6 H), 2.20–3.20 (m, 8 H), 3.53, 3.57 (2 s, 3 H), 3.60 (m, 3 H), 3.80–4.80 (m, 10 H), 6.80–8.65 (m, 18 H); exact mass calcd for $C_{46}H_{69}N_8O_{11}$ $(M + H)^+$ 909.5086, found (HR-FAB MS) 909.5087.

P$_2$-P$_3$′ HEA-Containing Peptide Inhibitors. (2*R*/*S*,3*S*)-3-[[*N*-(Benzyloxycarbonyl)asparaginyl]amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalanine methyl ester)-butane (CbzAsn[Phe-HEA-Pro]IlePheOMe) (29). The title compound (260 mg, 57% yield) was prepared from BocProIlePheOMe (310 mg, 0.633 mmol) by a two-step procedure. N-Deprotection according to general procedure C was followed by reaction with CbzAsnPheCH$_2$Cl (**8b**) (254 mg, 0.57 mmol) according to general procedures G and H: TLC R_f 0.25 (10% of MeOH–$CHCl_3$), 0.48 (4:1:1 *n*-BuOH–AcOH–H_2O); ^{1}H NMR (DMSO-d_6) δ 0.85 (m, 6 H), 0.95 (m, 1 H), 1.20–2.20 (m, 6 H), 2.20–3.20 (m, 8 H), 3.30–4.00 (m, 4 H), 3.54, 3.57 (2 s, 3 H), 4.10–6.65 (m, 4 H), 5.02 (m, 2 H), 5.35 (m, 1 H), 6.90 (bs, 1 H), 7.10–7.40 (m, 17 H), 7.45–8.70 (m, 4 H); exact mass calcd for $C_{43}H_{57}N_8O_9$ $(M + H)^+$ 801.4187, found (HR-FAB MS) 801.4183.

(2*R*/*S*,3*S*)-3-[[*N*-(2-Quinolylcarbonyl)asparaginyl]amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalanine methyl ester)butane (QuaAsn[Phe-HEA-Pro]IlePheOMe) (31). The title compound (21 mg, 47% yield) was prepared from CbzAsn[Phe-HEA-Pro]IlePheOMe **29** (43.6 mg, 0.054 mmol) by a two-step procedure. N-Deprotection was carried out according to general procedure I, except using ethanol (5 mL) as solvent together with *p*-toluenesulfonic acid (10 mg, 0.054 mmol). The catalyst was removed by filtration and the filtrate concentrated to dryness. The residue was coupled with 2-quinolinecarboxylic acid (10.5 mg, 0.0594 mmol) according to general procedure B: TLC R_f 0.26 (10% of MeOH–$CHCl_3$), 0.45 (4:1:1 *n*-BuOH–AcOH–H_2O); ^{1}H NMR ($CDCl_3$) δ 0.90 (m, 6 H), 1.00–1.50 (m, 2 H), 1.60–2.40 (m, 6 H), 2.40–3.30 (m, 10 H), 3.63, 3.72 (2 s, 3 H), 3.57–3.75 (m, 1 H), 4.10 (m, 1 H), 4.30 (m, 1 H), 5.85

(m, 3 H), 5.40–6.40 (m, 3 H), 6.60–7.40 (m, 11 H), 7.55–9.10 (m, 8 H); exact mass calcd for $C_{45}H_{56}N_7O_8$ $(M + H)^+$ 822.4190, found (HR-FAB MS) 822.4171.

(2*S*,3*S*)-3-[[*N*-(Benzyloxycarbonyl)asparaginyl]amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalaninol)-butane (CbzAsn[Phe-HEA-Pro]IlePhe-ol) (38). CbzAsn-[Phe-(*S*)-HEA-Pro]IlePheOMe (40 mg, 0.05 mmol) was dissolved in MeOH (4 mL) and cooled to 0 °C. $NaBH_4$ (23 mg, 0.60 mmol) was added and the reaction mixture stirred at 0 °C for 4 h. An additional portion of $NaBH_4$ (23 mg, 0.60 mmol) was added and the reaction was stirred for another 2 h, the course of reaction being monitored by TLC. The reaction mixture was quenched with water (10 mL) and extracted with EtOAc (30 mL × 3). The combined EtOAc extracts were washed with water and brine and dried over $MgSO_4$. The product was purified by silica gel chromatography using 3–4% MeOH in $CHCl_3$: TLC R_f 0.18 (10% MeOH–$CHCl_3$); 0.46 (4:1:1 *n*-BuOH–AcOH–H_2O); $[\alpha]^{25}_D$ −70.5° (*c* 0.4, DMSO); ^1H NMR (DMSO-d_6) δ 0.70–0.89 (m, 6 H), 1.00 (m, 1 H), 1.30 (m, 1 H), 1.70 (m, 4 H), 2.05 (m, 1 H), 2.20–3.15 (m, 11 H), 3.35 (m, 2 H), 3.65 (m, 1 H), 3.90–4.20 (m, 4 H), 4.30 (m, 1 H), 4.82 (m, 1 H), 5.02 (s, 2 H), 5.33 (m, 1 H), 6.88 (bs, 1 H), 7.10–7.38 (m, 19 H), 8.05 (m, 1 H); calcd for $C_{42}H_{57}N_8O_8$ $(M + H)^+$ 773, found (HR-FAB MS) 773.

***N*-(*tert*-Butoxycarbonyl)phenylalanine Methylamide (BocPheNHMe).** The title compound (1.23 g, 88% yield) was prepared from Boc-phenylalanine (1.33 g, 5.0 mmol) and methylamine hydrochloride (0.38 g, 5.50 mmol) according to general procedure B. The compound was used without further purification: ^1H NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9 H), 2.73 (d, J = 5.0 Hz, 3 H), 3.06 (d, J = 6.0 Hz, 2 H), 4.28 (q, J = 6.0 Hz, 1 H), 5.05 (bs, 1 H), 5.75 (bs, 1 H), 7.22 (m, 5 H).

***N*-(*tert*-Butoxycarbonyl)isoleucinylphenylalanine Methylamide (BocIlePheNHMe).** The title compound was prepared from Boc-phenylalanine methylamide (1.23 g, 4.42 mmol) by two-step reactions, first N-deprotection according to general procedure C, followed by coupling with Boc-isoleucine hemihydrate (1.03 g, 4.42 mmol) according to general procedure B. The crude product was used directly for the next reaction: TLC R_f 0.19 (50% EtOAc–hexane), 0.55 (10% MeOH–$CHCl_3$).

***N*-(*tert*-Butoxycarbonyl)prolylisoleucinylphenylalanine Methylamide (BocProIlePheNHMe).** The title compound (1.88 g, 87% yield from BocPheNHMe) was prepared by two-step reactions, first N-deprotection of BocIlePheNHMe (4.40 mmol) according to general procedure C, followed by coupling with Boc-proline (0.95 g, 4.40 mmol) according to general procedure B. The product was purified by flash chromatography using 3% MeOH in $CHCl_3$: TLC R_f 0.06 (50% EtOAc–hexane), 0.35 (10% MeOH–$CHCl_3$); $[\alpha]^{25}_D$ −76.6° (*c* 0.7, MeOH); ^1H NMR (300 MHz, $CDCl_3$) δ 0.90 (m, 6 H), 0.91–1.10 (m, 2 H), 1.46 (s, 9 H), 1.92 (m, 3 H), 2.12 (m, 2 H), 2.75 (d, J = 5.0 Hz, 1 H), 3.45 (m, 3 H), 4.05–4.20 (m, 2 H), 4.85 (m, 1 H), 6.65 (bs, 1 H), 6.78 (d, J = 8.0 Hz, 1 H), 6.92 (bs, 1 H), 7.20 (m, 5 H).

(2*R*/*S*,3*S*)-3-[[*N*-(Benzyloxycarbonyl)asparaginyl]amino]-2-hydroxy-4-phenyl-1-(*N*-prolylisoleucinylphenylalanine methylamide)butane (CbzAsn[Phe-HEA-Pro]IlePheNHMe) (33). The title compound (220 mg, 51% yield) was prepared from BocProIlePheNHMe (293 mg, 0.60 mmol) by a two-step procedure. N-Deprotection according to general procedure C was followed by reaction with CbzAsnPheCH$_2$Cl (**8b**; 240 mg, 0.54 mmol) according to general procedures G and H: TLC R_f 0.20 (10% MeOH–$CHCl_3$); ^1H NMR (DMSO-d_6) δ 0.80–0.85 (m, 6 H), 0.95 (m, 1 H), 1.10 (m, 1 H), 1.70 (m, 3 H), 2.05 (m, 1 H), 2.20–3.20 (m, 14 H), 3.30–4.70 (m, 7 H), 5.00 (m, 2 H), 5.40–5.70 (m, 1 H), 6.90 (m, 1 H), 7.00–7.40 (m, 16 H), 7.70–8.40 (m, 4 H); exact mass calcd for $C_{43}H_{58}N_7O_8$ $(M + H)^+$ 800.43469, found (HR-FAB MS) 800.4371.

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