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Development of HIV fusion inhibitors¶

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Abstract: In the past 25 years, the worldwide AIDS epidemic has grown such that roughly 38 million people were estimated to be living with the disease worldwide at the end of 2003. The introduction of antiretroviral-based therapies, beginning in 1987, has enabled many to live with HIV as a chronic, rather than terminal, disease. However, the emergence and spread of drug-resistant strains highlights the continued need for new therapies with novel modes of action. In 2003, the FDA and EMEA approved enfuvirtide (Fuzeon), a 36 amino acid peptide derived from the natural gp41 HR2 sequence, as the first HIV fusion inhibitor. T-1249, a 39 amino acid fusion inhibitor, is active against viruses that develop resistance to enfuvirtide. The development of FIs and the processes to manufacture enfuvirtide and T-1249 on an unprecedented scale for peptide therapeutics are presented. Synthetic routes based on a combination of solid phase peptide synthesis and solution phase fragment condensation as well as the analytical controls necessary to insure a robust process are discussed. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fusion inhibitors; HIV; enfuvirtide; T-1249; peptide therapeutics

INTRODUCTION

HIV is thought to have first appeared in humans in western Africa sometime in the early to mid twentieth century [1,2]. It spread rapidly in the later half of that century, likely due to increased international travel, blood transfusions, and intravenous drug use, and became recognized as a global epidemic in the 1980s [3]. According to recent data, roughly 38 million individuals worldwide are living with HIV with 5 million new cases in 2003 [4]. The virus is currently spreading rapidly in the Caribbean, India, China, Thailand, and the former republics of the Soviet Union [5]. In the United States, where prevention and treatment greatly reduced the spread of the HIV in the 1990s, it is now appearing in new populations; African Americans (13% of the US population), accounting for about half of the new cases with a growing number of cases in African-American women [6].

The majority of deaths due to HIV are from opportunistic infections, which are nonlethal or easily treated in patients with strong immune systems, but deadly to those with AIDS, whose immune systems have been ravaged by HIV. Deaths due to AIDS were estimated at 3.1 million in 2004 bringing the total number of deaths to approximately 23 million since the beginning of the epidemic [7]. In the United States, deaths due to AIDS peaked in 1994–1995 at which point this was the leading cause of death among adults 25–44 years old [8]. Owing to improved treatment and management of the disease, this rank had dropped to 22nd in 2001 [9].

Less than 10 years after the AIDS epidemic hit the world stage, the first antiretroviral (ARV) therapy was introduced. Since that time, 23 additional drugs/drug combinations have been approved for the treatment of HIV [10]. These are broken down into four classes based upon the type of compound and mechanism of action. Zidovudine (Retrovir, AZT), the first ARV, was approved in 1987 and belongs to the nucleoside reverse transcriptase inhibitor (NRTI) class. Problems with this class of compounds were quickly noted, including toxicity, low potency, and the emergence of drug-resistant virus. Further research led to the protease inhibitors (PI), pioneered by saquinavir mesylate (Invirase) in 1995, and the nonnucleoside reverse transcriptase inhibitors (NNRTI), led by nevirapine (Viramune), in 1996.

The initial three classes of ARVs are all small molecules that act in the cytoplasm to block critical stages of the HIV life cycle. As their names imply, NRTIs block the action of the enzyme responsible for reverse transcription of the viral RNA into cDNA. The NRTIs are incorporated into the growing DNA strand, but, as they lack a 3′ hydroxyl group, cause termination of the

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growing oligonucleotide. The NNRTIs bind adjacent to the active site of the RT enzyme, causing conformational changes that reduce the activity of RT. The PIs block cleavage of maturing viral proteins by binding in the protease active site. New viral particles are still produced, but, lacking mature viral proteins, they are no longer infective.

The development of drug resistance noted with zidovudine has also been a critical problem with all other drugs from the NRTI, PI, and NNRTI classes and leads to rapid failure of ARV monotherapy. The introduction of combination therapy (2 ARVs) and highly active antiretroviral therapy (HAART, 3 or more ARVs) in 1997 greatly improved the prognosis for HIV patients, but has led to the selection of multidrug resistant virus in which mutations accumulate in the virus that make it unresponsive to entire classes of ARVs [11]. This problem is often compounded by poor compliance to the complicated treatment regimen. The issue of resistance is illustrated by a report, which studied AIDS patients in the late 1990s and found that 48% of the study population was resistant to drugs from two ARV classes and 13% was resistant to drugs from all three classes in use at the time [12]. Managing the treatment of individuals infected with resistant virus is quite challenging and prone to failure over time [13]. A crucial addition to the arsenal of ARV medications was the introduction of enfuvirtide (Fuzeon, ENF; formerly known as T-20 and DP-178), the first member of the fourth class of ARVs, the fusion inhibitors (FI), which received fast-track approval and was launched in the United States and Europe in 2003 followed by full approval in 2004. Through its unique mode of action, enfuvirtide addresses many of the unmet needs of HIV regimens for treatment experienced patients such as activity against multidrug resistant virus, limited side effects, and absence of drug/drug interactions. In fact, a recently publicized strain of HIV is responsive (*in vitro*) to enfuvirtide while showing resistance to all other classes of ARVs [14].

FUSION INHIBITORS

Enfuvirtide is the first member of a broader class of ARVs termed *entry inhibitors*, which act extracellularly to block binding of the virus to the target cell and subsequent delivery of the viral RNA into the target cell [15–19]. In addition to fusion inhibitors, other entry inhibitors, which have yet to reach the market, include gp120, CCR5, and CXCR4 inhibitors. Viral entry is a complex process beginning with binding of the viral envelope protein gp120 to CD4 receptors on the target cell. This event is followed by binding to a coreceptor, CXCR4 or CCR5. These interactions induce a conformational change on gp120, which exposes the buried transmembrane protein gp41. This protein comprises an *N*-terminal fusion peptide (FP) domain responsible for attachment to the target cell membrane, two heptad repeat regions HR1 and HR2, and a transmembrane region that anchors the protein to the viral membrane. After exposure of gp41, the HR2 region zips around a preformed trimer of HR1 regions in an antiparallel fashion to form a six-helix bundle. This conformational change in gp41 brings the viral and

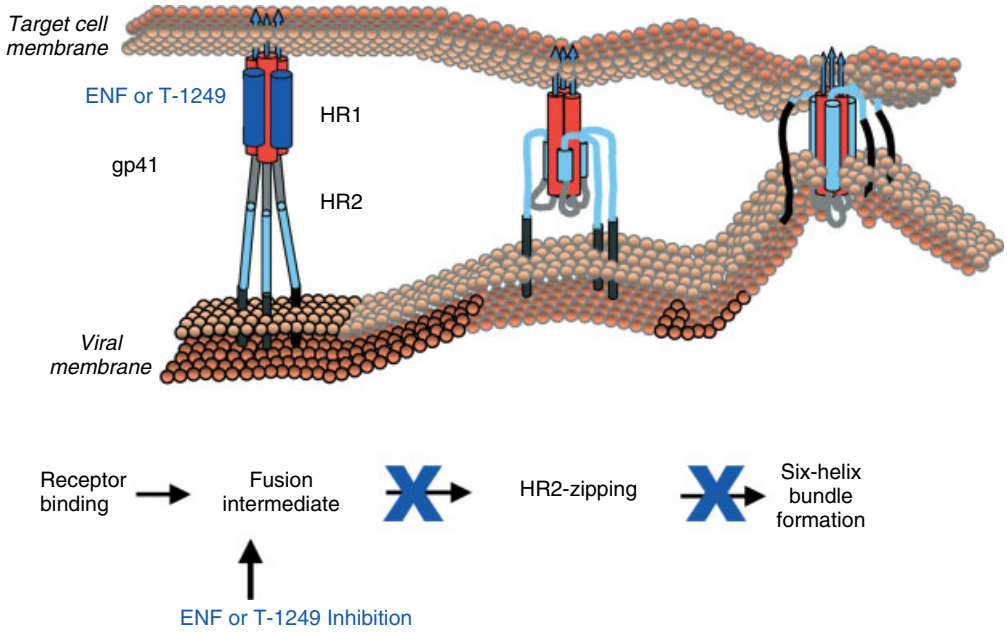


Figure 1 Fusion inhibition by enfuvirtide and T-1249. Fusion of the viral and target cell membranes requires a conformational change involving zipping of the HR2 regions of gp41 around the HR1 regions into a six-helix bundle. Binding of a fusion inhibitor (blue cylinders) to the HR1 region (red cylinders) blocks HR2 zipping and prevents fusion.

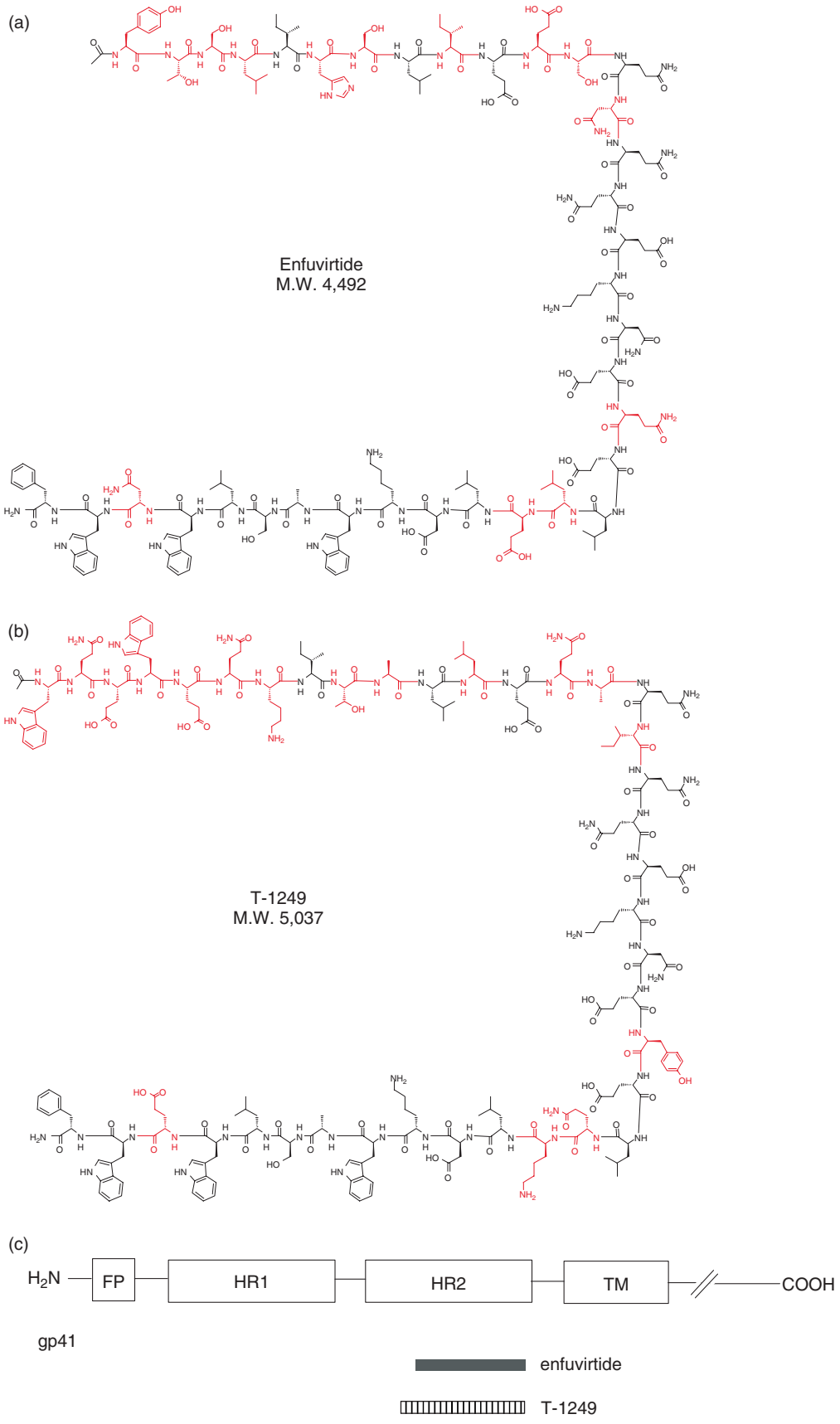


Figure 2 Chemical structures of (a) enfuvirtide, MW 4492 and (b) T-1249, MW 5037 (conserved amino acids are shown in black. Variable amino acids are shown in red). (c) The relationship between enfuvirtide and T-1249 and gp41. Gp41 comprises an *N*-terminal fusion peptide (FP), two heptad repeat peptides (HR1 and HR2), and a *C*-terminal transmembrane peptide (TM).

target cell membranes in close proximity and allows fusion to proceed. However, since this zipping process takes several minutes to occur, an opportunity exists to block this process, and cell infection via competitive inhibition [20]. It is in this capacity that the fusion inhibitors enfuvirtide and T-1249 act to block HIV (Figure 1).

Enfuvirtide (Figure 2a) is a 36 amino acid peptide representing a portion of the natural sequence of the HR2 region from the gp41 of the HIV-1 LAI strain. T-1249 (Figure 2b) is a designed 39 amino acid peptide derived from the HR2 regions of HIV-1 LAI, HIV-2 NIHZ, and SIV mac251. Both peptides are acetylated on the *N*-terminus and amidated on the *C*-terminus to improve stability. T-1249 shares approximately 60% sequence homology with enfuvirtide. Both enfuvirtide and T-1249 are highly potent and specific inhibitors of HIV fusion *in vitro* and have also been shown to be effective antiviral agents *in vivo*.

The goal of treatment in patients with prior drug exposure and drug resistance is to reestablish maximal virus suppression [21]. Adding a drug with a new mechanism of action (e.g. an entry inhibitor) to an optimized background regimen can add significant ARV activity, particularly if at least one other active drug is present. In the pivotal phase III TORO studies with a highly ARV treated population, many of whom no longer had an active drug in their optimized background, patients in the enfuvirtide-containing arm were significantly less likely to experience virologic failure after 24 weeks of treatment (41.7%) than the control group (64.2%) (Figure 3) [22]. At 96 weeks, patients in the enfuvirtide arm were twice as likely to have HIV-1 RNA counts below 400 copies/ml than patients from the control arm (containing an optimized background regimen, but not enfuvirtide) at 48 weeks [23]. Additionally, 31% of the patients in the enfuvirtide arm demonstrated an increase in CD4 cell counts of at least 100 cells/mm^3, whereas only 10% of the control arm met this mark. These unprecedented results in heavily treatment experienced patients were further extended in two recent studies where enfuvirtide was combined with investigational PI's to drive the virus to undetectable levels (<50 copies/ml) in even higher proportions of patients exhibiting triple class resistance [24,25]. In particular, a response typical of treatment naive patients was observed in a highly ARV-treated population when given a regimen containing enfuvirtide and TMC-114/ritonavir (67% reached achieved undetectable HIV levels) [24]. This study further illustrates the importance of constructing regimens containing fully active drugs from different classes to maximally suppress virus in treatment-experienced patients.

As with other ARVs, resistance to enfuvirtide can develop when the drug is used without sufficient activity in the background regimen. Unlike some ARVs whose antiviral activity is completely nullified by a single mutation, resistance to enfuvirtide *in vitro* occurs as a continuum of response where different mutations decrease sensitivity to varying degrees and multiple mutations are required for the most significant losses of activity [26]. Mutations that confer resistance to enfuvirtide also exact a toll on viral fitness such that enfuvirtide-resistant viruses exhibit significant reductions in replication capacity *in vitro* [27]. Consistent with these observations on fitness deficits in enfuvirtide resistant virus, discontinuation

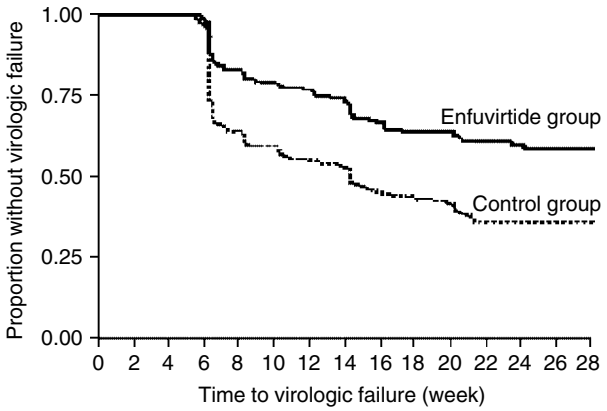


Figure 3 Comparison of time to protocol-defined virologic failure of enfuvirtide-containing (180 mg/day) and control arms of Phase III TORO 1 study. Analysis based on intent to treat model where patients who discontinue or switch treatment are classified as failures. Used with permission from Lalezari, *et al. N. Eng. J. Med.* **2003**, *348*, 2175–2185. Copyright ©2003 Massachusetts Medical Society. All rights reserved.

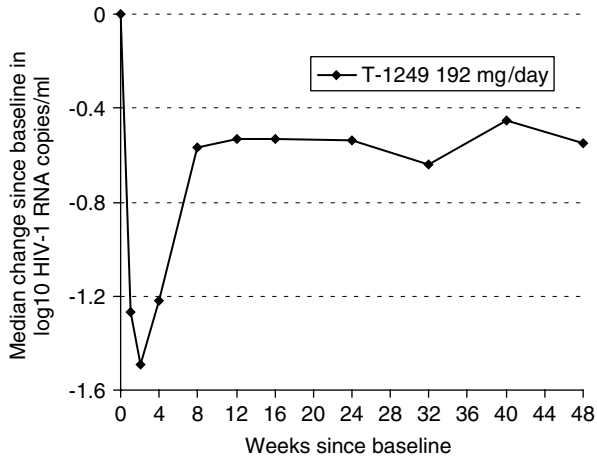


Figure 4 Median change in $\log_{10}$ HIV RNA copies/ml from baseline by study week in patients who switched to a T-1249 (192 mg/day) containing regimen in study T-1249-105 after failing an enfuvirtide (180 mg/day) containing regimen (>5000 HIV-1 RNA copies/ml). Adapted from Lalezari J, Zhang Y, DeMasi RA, Salgo M, Miralles GD. Long term safety of T-1249, a potent inhibitor of HIV fusion. 44th ICAAC, Washington, DC, 2004, H-210.

of enfuvirtide treatment in patients who have developed resistance has been observed to result in a return of circulating wild-type virus after a period of about 4 months [28,29]. In addition, fusion inhibitors can be designed to overcome resistance to enfuvirtide as demonstrated with T-1249 in studies T1249-102 and T1249-105 conducted in patients who developed resistance and were failing an enfuvirtide-containing regimen (Figure 4) [30,31].

ENFUVIRTIDE/T-1249 ROUTE

From the outset, it was questioned whether large peptides such as enfuvirtide (MW 4492) and T-1249 (MW 5037) could be manufactured in a cost-effective manner on a multi-ton scale. Though many peptide therapeutics have reached the market and more are expected to be approved in coming years, the majority of these are significantly smaller than enfuvirtide and, more importantly, their annual production requirements are typically less than 10 kg [32,33]. In contrast, enfuvirtide is produced on the metric ton scale.

Currently marketed peptide drugs are manufactured by a variety of routes, including pure solution or solid phase synthesis and biological expression in a variety of cell lines. For the early preclinical and phase I clinical supply of enfuvirtide and T-1249, a linear solid phase synthesis (route 1) was sufficient. The Rink amide MBHA resin and Fmoc-protected amino acids with acid labile (Boc, trityl, and *t*-butyl) side chain protecting groups were used. Typical crude peptide purities from this route were 30–40A% with overall yields of less than 10%.

An improved route was needed for later-stage clinical trials and for manufacturing. A route 2 synthesis (Figure 5) was developed on the basis of the solid phase synthesis of three protected peptide fragments followed by their assembly into the final product in solution. The peptides are split into three roughly equivalent fragments based on their ease of assembly, physical properties, and propensity for racemization at the disconnect sites. These fragments are built independently on the solid phase and then released into solution (steps 1a–c). The peptide is then assembled from the *C*- to *N*-terminal fragments in solution (steps 2–4) followed by a global side chain deprotection (step 5) and purification (step 6). The same Fmoc-protected amino acids used in the route 1 synthesis were kept as starting materials, simplifying the comparison of material produced by the route 1 and route 2 methods. The super-acid labile 2-chlorotrityl chloride resin (2-CTC) was chosen as a base for the solid phase synthesis because of its ease of cleavage to protected peptide fragments under mildly acidic conditions, the lack of racemization during loading of the first amino acid, and the option of recycling the resin. Furthermore, it was projected that competitive bidding among multiple suppliers would ensure an adequate supply of this resin at an acceptable cost on the manufacturing scale.

The route 2 solid phase chemistry for T-1249 (Scheme 1) is based on the enfuvirtide synthesis, which has been

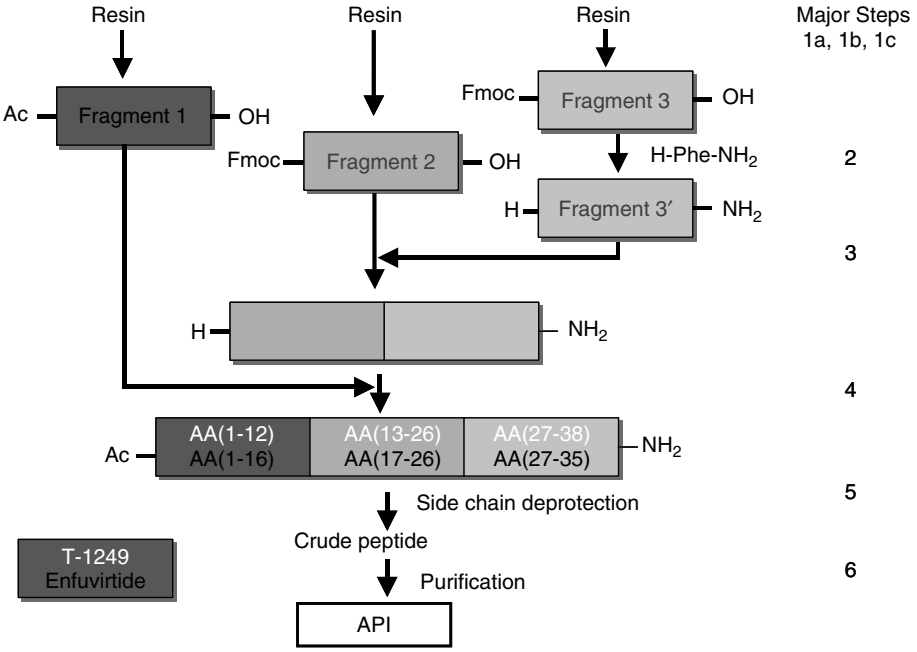
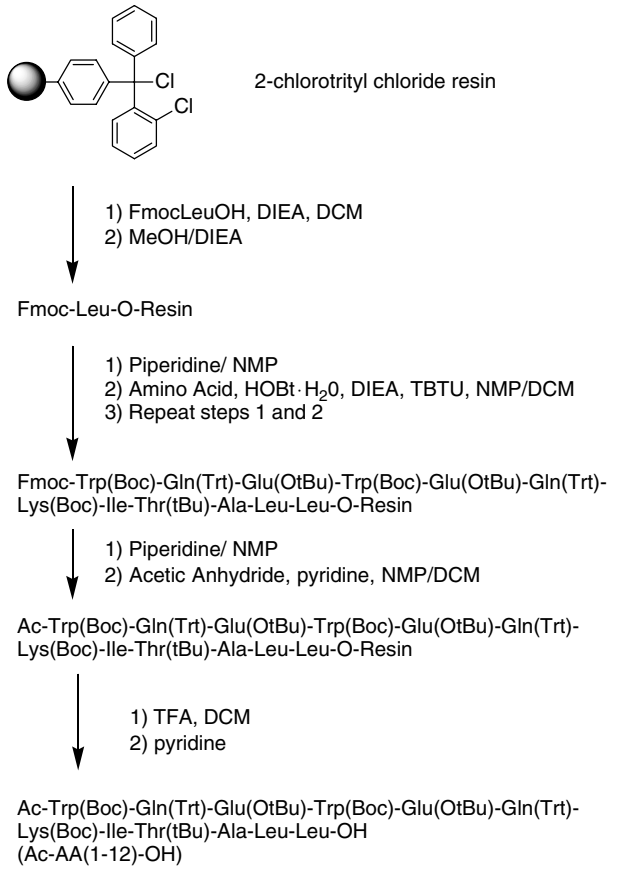


Figure 5 Schematic representation of the route 2 syntheses of Fuzeon and T-1249. The peptides are split into three fragments, which are assembled on 2-CTC resin (step1). After cleavage, the *C*-terminal phenylalanine amide is coupled with fragment 3 (step 2) and the full-length peptides are assembled in solution (steps 3 and 4). After a global side chain deprotection (step 5), the API is purified by RP HPLC (step 6) and isolated by precipitation.



Scheme 1 Solid phase synthesis of T-1249 fragment Ac-AA(1–12)-OH.

previously described [32]. The *C*-terminal amino acid of each fragment is loaded on the 2-CTC resin in DCM in the presence of excess Hunig's base to a level that will allow the fragment to be synthesized in optimum purity and yield with maximum throughput. Unreacted functional sites are capped with methanol, again in the presence of excess Hunig's base. The fragment is then built using standard solid phase chemistry by first removing the *N*-terminal Fmoc group with piperidine in NMP and then adding a solution of the next Fmoc amino acid that has been preactivated with TBTU/6-Cl HOBt/DIEA in a mixture of NMP and DCM. For the *N*-terminal fragment (AA1–12 in T-1249), the *N*-terminus is acetylated with acetic anhydride and pyridine. The fragments are then cleaved from the resin using cold 1–2% TFA in DCM. The TFA is quenched with pyridine and the fragment is washed from the resin with DCM. After switching to an alcoholic solvent by evaporation of the DCM, the peptide fragments are precipitated by the addition of water and isolated in high yield and purity by filtration.

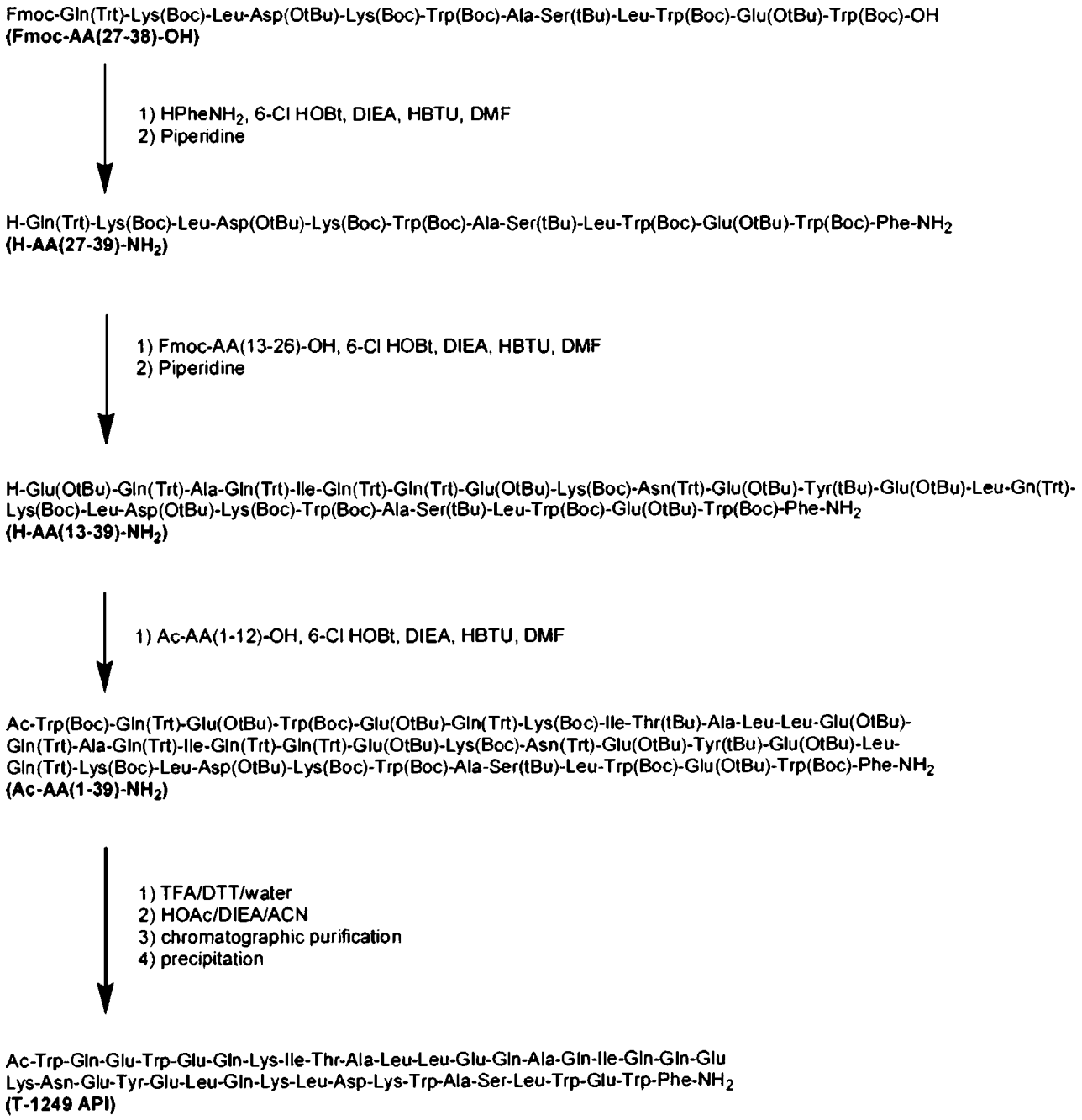
The solution phase assembly of the full-length peptide begins with the coupling of H-Phe-NH_2 to the *C*-terminus of Fmoc-AA(27–38)-OH in DMF using TBTU/6-Cl HOBt/DIEA. The Fmoc group is removed *in situ* and the amine product is isolated by precipitation with water. After washing to thoroughly remove residual piperidine and piperidine-dibenzylfulvene adducts, the H-AA(27–39)-NH_2 is coupled with Fmoc-AA(13–26)-OH in a similar manner. An *in situ* Fmoc removal is again used allowing isolation of H-AA(13–39)-NH_2 by precipitation with water. Again, after thoroughly removing the piperidine by-products, the full-length peptide is achieved by coupling with Ac-AA(1–12)-OH. The resulting Ac-AA(1–39)-NH_2 may be isolated by precipitation or separated from the reaction mixture by an extraction procedure. Either way, the material is then subjected to a global side chain deprotection using TFA : DTT : water and precipitated with cold MTBE. This intermediate is decarboxylated as a slurry in ACN or IPA buffered to pH 4–5 with DIEA and acetic acid. The resulting crude T-1249 is purified in a single pass by reversed phase HPLC and isolated by precipitation near its isoelectric point in high yield and purity (Scheme 2).

The process described above is carried out in conventional plant equipment with minimal isolations and no lyophilization required. No single-source proprietary resins or reagents are used allowing them to be subjected to competitive bidding and minimizing supply issues. Switching to the route 2 synthesis approximately doubled the purity of the crude peptide and tripled the overall synthetic yield. In addition, risk was averted while scaling the process since a failure to meet specifications for one fragment could be detected and corrected without jeopardizing an entire campaign. These improvements from an optimized process, coupled with the drop in raw material costs for CTC resin and Fmoc amino acids due to economies of scale, allowed enfuvirtide to meet its commercial manufacturing requirements.

PROCESS CONTROL

Control of the process described above is a critical component of achieving a cost-effective large-scale synthesis of large peptides and is required to maintain the level of reproducibility necessary for process validation. Thus, considerable effort has been expended to develop analytical methods for monitoring all stages of the process. In the solid phase portion, the initial amino acid loading levels are accurately determined by HPLC and/or GC methods and the couplings and deprotections are monitored by qualitative Kaiser tests for the presence/absence of free amines and the chloranil test for the presence of residual piperidine [34–36]. HPLC monitoring of the couplings and deprotections is possible, but not routinely used. All aspects of the solution phase steps are monitored by HPLC.

In addition to their utility in monitoring reaction progress, these analytical methods are used to identify and track impurities, ensure process control by



Scheme 2 Solution phase assembly of T-1249.

assessing the impact of process, equipment, and scale changes, and to ensure the quality of the intermediates and final product.

The process has been examined carefully for areas of potential impact on quality and throughput. Resin loading can be affected by incomplete endcapping or a loading level that is above or below the optimum range or nonhomogeneous throughout the batch. Deletions, double additions, racemization, and premature cleavage may impact quality and yield during solid phase synthesis and loss of side chain protecting groups, esterification, and aspartimide formation may occur during cleavage from the solid support. During solution phase assembly, racemization, tetramethylguanidinium adduct formation, piperidine adduct formation, and incomplete couplings and deprotections may occur. Finally, incomplete protecting group removal or migration, TFA-induced degradation, and deamidation may occur during side chain deprotection and peptide purification. To aid in the identification of impurities and in the development of the analytical methods used for process control, a large set of impurity markers were synthesized for both enfuvirtide and T-1249 (Table 1).

Table 1 Description of impurity markers synthesized for T-1249 and number of markers resolved by HPLC. (A) Solid phase synthesis single site fragment impurity markers. (B) Solution phase fragment impurity markers (C) API impurity markers

A

Fragment	Impurity type	# of markers	# resolved by HPLC
Ac-AA(1–12)-OH	Deletion	11	11
	Double hit	11	11
	Racemization	12	ND
	Ester	1	1
Fmoc-AA(13–26)-OH	Deletion	12	11
	Double hit	12	11
	Racemization	13	ND
	Ester	1	1
Fmoc-AA(27–38)-OH	Deletion	11	11
	Double hit	11	11
	Racemization	12	ND
	Ester	1	1

ND, not determined.

B

Fragment	Impurity type	Resolved by HPLC?
H-AA(27–39)-NH_2	Piperidine adduct	Yes
	Tetramethyl guanidinium adduct	Yes
	Incomplete coupling	Yes
	Incomplete de-Fmoc	Yes
	Racemization	Yes
H-AA(13–39)-NH_2	Piperidine adduct	Yes
	Tetramethyl guanidinium adduct	Yes
	Incomplete coupling	Yes
	Incomplete de-Fmoc	Yes
	Racemization	Requires scdp
Ac-AA(1–39)-NH_2	Piperidine adduct	Yes
	Tetramethyl guanidinium adduct	Yes
	Incomplete coupling	Yes
	Racemization	Requires scdp

Scdp, side chain deprotection.

C

Type	# synthesized	# resolved by HPLC
Single site deletions	36	35
Single site double hits	34	34
Single site racemization	36	34
Deamidation	9	8
Aspartimide	2	2
Ile/Leu switches	7	3
Others	4	4

CONCLUSIONS

The Fusion Inhibitors (FI), represented by enfuvirtide and T-1249 represent a novel class of compounds for the treatment of HIV. These peptides work by a unique mode of action that blocks the virus from entering the target cell. They lack systemic toxicity and cross-resistance to other existing ARVs. However, the size and complexity of these molecules has necessitated the development of peptide synthesis to an unprecedented scale. The enfuvirtide commercial production process has demonstrated that large peptides can be manufactured cost-effectively on a multi-ton scale by chemical synthesis so long as the chemistry is efficient and robust, the starting materials and reagents are subjected to competitive bidding, and stringent process control is adhered to. This process was validated in September 2002 and meets the commercial production requirements with respect to quality and cost. The application of an enfuvirtide-like process to T-1249 clinical manufacturing has further demonstrated its utility to other therapeutically relevant peptides. The efficient manufacture of enfuvirtide and T-1249 paves the way for the development of the next generation of Fusion Inhibitors, which promise to be more potent, easier to administer, and possess a high genetic barrier to resistance, and will solidify peptide FIs as a backbone of therapy for the treatment experienced HIV patient.

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