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Process Development of TRI-999, a Fatty-Acid-Modified HIV Fusion Inhibitory Peptide

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Abstract:

TRI-999 is a HIV fusion inhibitory peptide designed to improve upon the efficacy and convenience of Enfuvirtide (ENF, Fuzeon), the first approved HIV entry inhibitor. Derived from a gp41 HR2 region partially overlapping the ENF sequence, TRI-999 is modified using a fatty acid conjugation strategy through a poly(ethylene glycol) (PEG) linker to maintain potency while achieving the desired PK. Synthetic route comparison, process development, and GMP manufacturing at the kilo scale of TRI-999 are reported.

Introduction

Enfuvirtide (ENF, Fuzeon, formerly T20) is the first approved HIV fusion inhibitor,[1] as well as the first HIV entry inhibitor. Its mechanism of action is different from existing anti-HIV drugs in that the existing antiretrovirals work inside T cells to stop virus replication, but Enfuvirtide works extracellularly where it blocks fusion, a late stage of the entry process. This unique function gives fusion inhibitors (FI) the potential to treat multiple-class-resistant strains of HIV.[2] Additionally, fusion inhibitors are less likely to cause unwanted side effects or interfere with other drugs in an antiviral regimen. However, as with all current HIV medications, continued exposure, especially if the virus is not completely suppressed, can lead to emergence of viral resistance to Enfuvirtide, though HIV can still be partially suppressed and CD4 cell counts increased even if there is resistance.[3] Enfuvirtide is administered as a twice-daily subcutaneous injection. The inconvenience and potential for injection site reactions (ISRs) of this route of administration has slowed the acceptance of Enfuvirtide by patients and their physicians.[4] The discovery and development of fusion inhibitors with improved efficacy, resistance profiles, and convenience of administration are needed.

TRI-999 (Figure 1) is a HIV fusion inhibitory peptide with improved potency, generic barrier to resistance, and pharmacokinetic (PK) properties, relative to ENF, that could enable much less frequent administration with the help of improved formulation and delivery technologies. TRI-999 is derived from TRI-899, a 36 amino acid peptide corresponding to a portion of the natural sequence of the HR2 region from the HIV-1_{LAI} gp41, where 24 amino acid residues overlap with Enfuvirtide. The two methionine residues (position 1 and 4, Figure 2) have been replaced with leucine and glutamine, respectively. TRI-899 has very potent in vitro antiviral activity and a high genetic barrier to resistance but poor pharmacokinetic properties. Fatty acid conjugation has been a favored approach for peptide modification with various purposes of better membrane permeability, immunogenicity, enzymatic stability, pharmacokinetic properties or secondary structure induction.[5–14] By introducing a C18 fatty acid through a short PEG3 (8-amino-3,6-dioxaoctanoyl) linker onto the ϵ amino group of the $lysine_{30}$ residue, the desired PK was achieved in TRI-999 while the potency and

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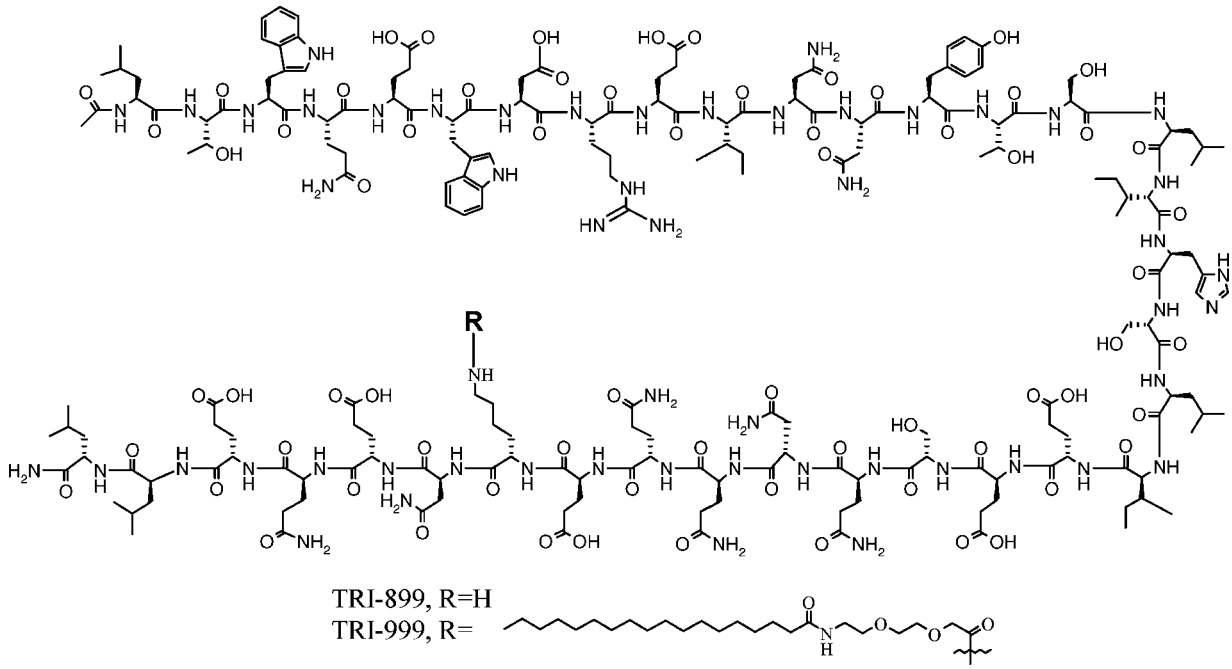

Figure 1. **Chemical structure of TRI-899 and TRI-999.**

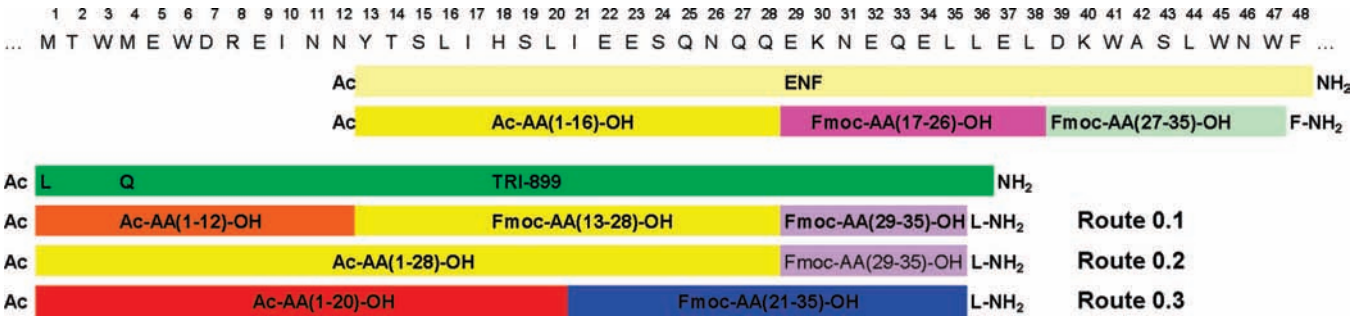

Figure 2. **Two- and three-fragment routes to TRI-899. The top sequence is amino acid residues 115–162 of the ectodomain of gp41 of the HIV1$_{LAI}$ clade, renumbered for easy reference; ENF sequence is listed in the yellow bar and its three-fragment synthesis is shown right below.**

resistance profile were maintained.[15,16] However, TRI-999 was not advanced to clinical development as it did not meet all the project criteria.

Original Research Routes

The selection of TRI-999 as a lead candidate came from screening numerous combinations of fatty acids and linkers. The base peptide TRI-899 was first synthesized to supply peptide conjugates for a structure–activity relationship (SAR) study.[16,17] Based on previous experience with ENF and T-1249,[18,19] several two- and three-fragment routes were explored to synthesize TRI-899 using the superacid-labile 2-chlorotrityl chloride resin (2-CTC) (Figure 2).

Sequence homology between ENF and TRI-899 enabled the use of an existing N-terminal fragment of ENF, Fmoc-AA(1–16)-OH, as the middle fragment (Fmoc-AA(13–28)-OH) of TRI-899, leaving the two relatively short fragments (Ac-AA(1–12)-OH and Fmoc-AA(29–35)-OH) to be synthesized. This three-fragment route (Route 0.1, Figure 2) provided rapid access to the initial supply of TRI-899.

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By extending this existing resin-bound N-terminal fragment of ENF to the N-terminus of TRI-899 and using the same C-terminal fragment (Fmoc-AA(29–35)-OH) from Route 0.1, a two-fragment route (Route 0.2, Figure 2) was outlined. ENF fragment, Fmoc-AA(1–16)-resin was successfully extended to yield Ac-AA(1–28)-OH and was isolated in a reasonable yield and purity with only one recouple (yield 84%, purity 79A%). The encouraging result inspired further examination of alternative two fragment routes since a successful two fragment approach could be more efficient than a three fragment approach due to a reduction in the number of isolations and impurities generated during solution phase synthesis (SPS).

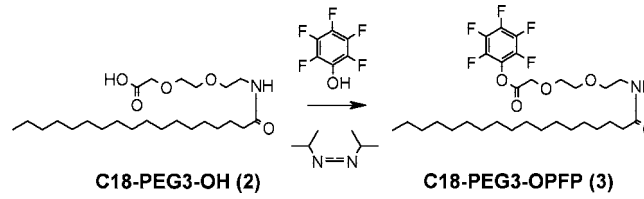
The presence of two leucine residues near the middle of TRI-899 presented attractive disconnect sites for two fragment approaches, since there is a good history with leucine disconnects from the synthesis of ENF and T-1249 and both fragments would share the same leucine-loaded resin. The fragments using Leu_{20} as the disconnect (Route 0.3, Figure 2) were built successfully in reasonable yields and purities (both above 80%) with no recouples. The solution characteristics of these fragments are also good, enabling assembly of the full length peptide in high yield and acceptable purity and conversion to crude TRI-899 with no issues (Route 1, Stage 4 in Table 1.). This synthesis was chosen for the initial scale up of TRI-899 and later became part of Route 1 of the TRI-999 synthesis.

Prior to and including the start of the first scale-up campaign, conjugation of TRI-899 with a fatty acid-PEG group was conducted by in situ activation with combinations of 6-Cl-HOBt/TBTU/DIEA (6-chloro-hydroxybenotriazole/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate/diisopropylethylamine) or HOAT/HBTU/DIEA (1-hydroxy-7-azabenzotriazole/*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate/diisopropylethylamine). The reactions were very rapid but gave relatively impure product (crude purity ~60A%). However, semipreparative HPLC purification could provide high purity conjugates (>90A%) in sufficient quantities for initial antiviral and PK SAR studies. The low yield appeared to be a combination of a lack of selectivity of the highly activated fatty acid linker species along with undesired activation of peptides by excess activators. More robust reaction conditions were required to achieve acceptable yield and purity during scale-up.

C18-PEG3-PFP Ester Coupling

One solution to the issue associated with in situ activation was to preactivate C18-PEG3-OH (**2**)[20,21] as its NHS (*N*-hydroxysuccinimidyl)[9] ester. Unfortunately, a significant amount of *N*-acylurea was generated when C18-PEG3-OH was converted to its NHS ester in the presence of dicyclohexylcarbodiimide (DCC). However, the use of pentafluorophenol (PFP) and diisopropylcarbodiimide (DIC) quantitatively furnished C18-PEG3-OPFP (**3**) without formation of *N*-acylurea (Scheme 1).[22]

Scheme 1. **Synthesis of C18-PEG3-OPFP**



The conjugation of TRI-899 TFA salt with C18-PEG3-OPFP in dimethylformamide (DMF) in the presence of excess DIEA was completed near quantitatively. The product was precipitated by the addition of MTBE and then reslurried in dichloromethane (DCM) prior to isolation by filtration. The reaction was specific enough that the product did not require purification to remove peptide impurities and residual C18-PEG3-OPFP was not detected in the product. However, a low level of residual C18-PEG3-OH and DIEA (0.1% w/w) were present. These impurities were removed by a polishing HPLC purification.

Route 1 Scale-Up Campaigns

Three campaigns were performed using the modified two-fragment convergent route (shown as Route 1 in Scheme 3), producing a total of 0.5 kg of TRI-999. This process served the purpose of providing material for formulation development and preclinical PK studies; however, the following technical issues were identified: (1) the salt exchange of TRI-899 after purification, (2) the isolation of TRI-899, and (3) the purification of TRI-999.

Because of the poor solubility of TRI-899 in an acidic environment, crude TRI-899 was purified in basic buffer (ammonium acetate−acetonitrile system). However, since the salt form of TRI-899 must be compatible with the C18-PEG3 conjugation reaction, a HPLC salt exchange was performed to convert TRI-899 to its TFA salt, which added one more HPLC operation and isolation to the process.

Continuing the trend of ENF and T-1249 toward implementing isolation by precipitation instead of lyophilization, precipitation of TRI-899 was examined in order to speed throughput and reduce energy and equipment costs. However, precipitated TRI-899 had reduced solubility in DMF, the solvent used in the C18-PEG3 conjugation reaction. Therefore TRI-899 remained isolated by lyophilization.

The residual DIEA and C18-PEG3-OH caused some toxicity concerns and required a rework via HPLC.

These three issues all related to the introduction of C18-PEG3-OH to the peptide. Thus a route change was considered to solve all three issues.

Two-Fragment C18-PEG3 "Incorporated" Routes

In the previous synthetic strategies, the fatty acid-PEG group was viewed as an appendage to the peptide that was introduced after peptide construction, enabling rapid derivatization during

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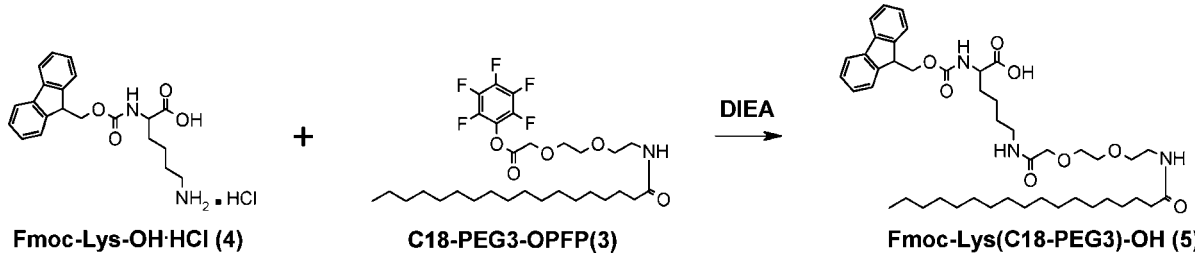
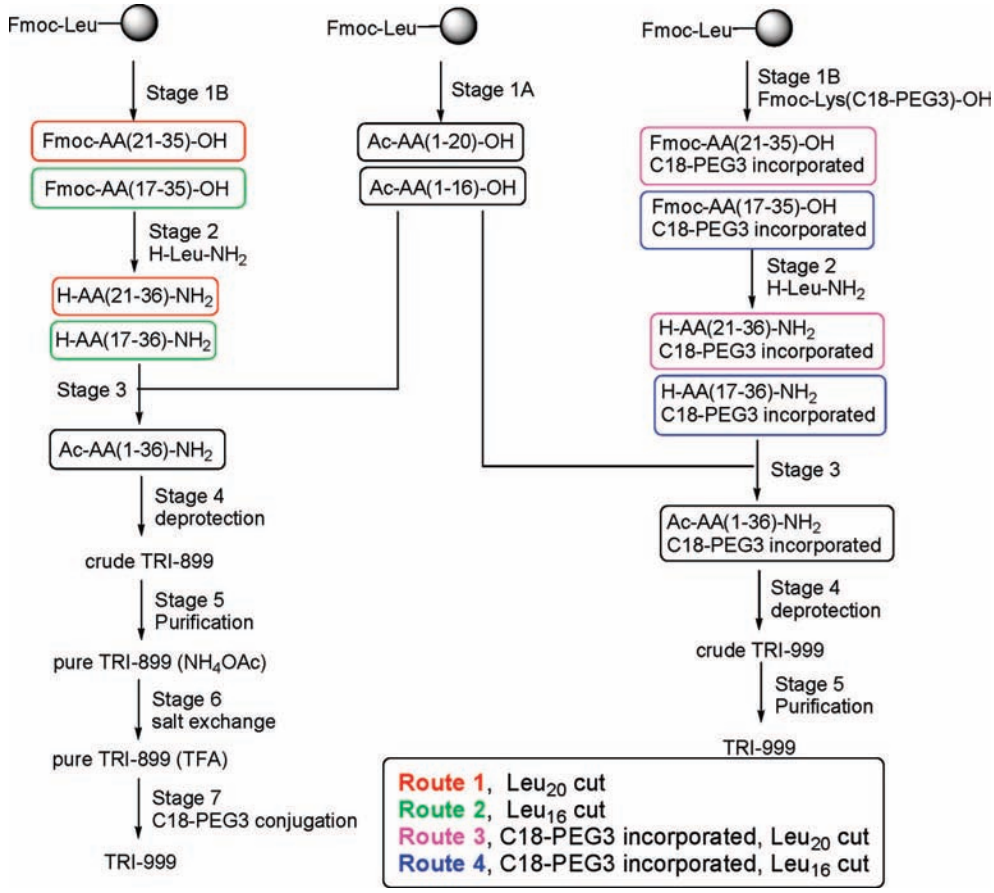
Scheme 2. **Synthesis of Fmoc-Lys(C18-PEG3)-OH**



Fmoc-Lys-OH·HCl (4) **C18-PEG3-OPFP(3)** **Fmoc-Lys(C18-PEG3)-OH (5)**

Scheme 3. **Original versus C18-PEG3 incorporated two-fragment routes on CTC resin**



precandidate selection. Once TRI-999 was chosen for further development, C18-PEG3 could be treated as a special protecting group on the ϵ amino group of lysine and incorporated in the solid-phase peptide synthesis (SPPS) of TRI-999 via a novel amino acid derivative, Fmoc-Lys(C18-PEG3)-OH (**5**). After completing the solution phase assembly, global side chain deprotection/decarboxylation, and purification, the final product would be TRI-999 with no need of further conjugation. This change would eliminate two HPLC steps and two lyophilization steps and allow for sourcing the lysine derivative. Potential complications of this "incorporated" strategy included (1) that the introduction of the long greasy fatty acid chain at an early stage of SPPS might impact the peptide chain elongation, (2) that this fatty acid chain may alter the physicochemical properties of the fragments and further impact the solution assembly of the full peptide, and (3) that it may alter the chromatographic behavior of the peptide and impurities, making it difficult if not impossible to purify the TRI-999 sufficiently. Nevertheless, the advantages over the postpeptide synthesis conjugation made this strategy worth pursuing.

The first stage of the "incorporated" route was to synthesize the building block, Fmoc-Lys(C18-PEG3)-OH (**5**). Fmoc-Lys-OH has very poor solubility in typical organic solvents such as DCM, THF (tetrahydrofuran), and DMF, except in the presence of HPFA (hexafluoroisopropanol), the acidic nature of which unacceptably slows the coupling reaction. Fortunately, however, the HCl salt of Fmoc-Lys-OH (**4**) is very soluble in DMF. Stoichiometric usage of DIEA neutralized the acid prior to the addition of C18-PEG-OPFP (**3**) to complete the conversion to Fmoc-Lys(C18-PEG3)-OH (**5**) (Scheme 2) in 83% yield and 97A% purity.

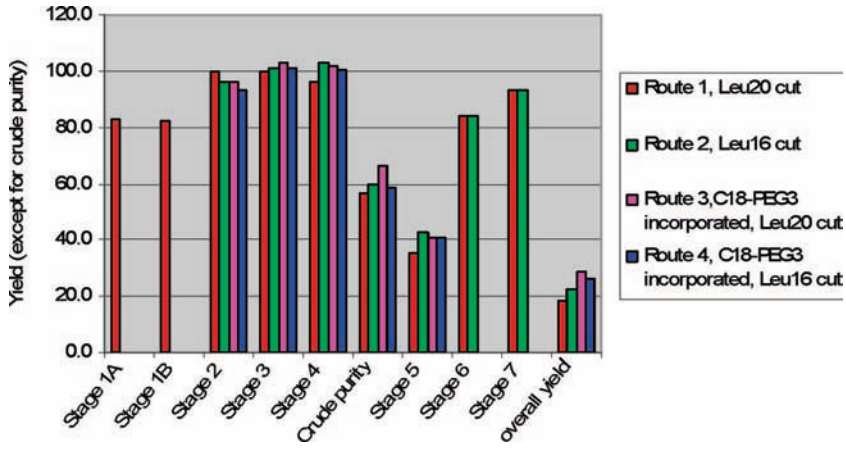
A set of routes (Scheme 3) were examined to compare the original two-fragment route (Route 1 in red) with the "incorporated" route (Route 3 in purple) and with alternate routes (Route 2 in green and Route 4 in blue) where the fragment disconnect was at Leu_{16} instead of Leu_{20}. This latter change

Table 1. **Comparison of C18-PEG3 incorporated versus nonincorporated Routes 1–4**

Route	description	stage	description	purity (%)	yield (%)	LCMS (expt)	LCMS (obsd)
1	Leu_{20} Cut	1A	Ac-AA(1–20)-OH	79.8[a]	83.0	4428.37	4428.48
		1B	Fmoc-AA(21–35)-OH	89.2	82.4	3955.99	3956.18
		2	H-AA(21–36)-NH_2	86.0	100.1	3846.18	3846.58
		3	Ac-AA(1–36)-NH_2	52.4	100.0	8256.38	8256.96
		4	**crude TRI-899**	**56.6**	96.3	4498.17	4498.00
		5	pure TRI-899 NH_4OAc salt	94.3	35.4	4498.17	4498.01
		6	pure TRI-899 TFA salt	93.5	84.0	4498.17	4498.02
		7	TRI-999	94.8	93.0	4409.50	4409.74
		overall			**18.2**		
2	Leu_{16} Cut	1A	Ac-AA(1–16)-OH	83.3	N/A[b]	3679.93	3679.36
		1B	Fmoc-AA(17–35)-OH	83.5	N/A[b]	4704.42	4705.18
		2	H-AA(17–36)-NH_2	84.6	96.1	4594.46	4594.88
		3	Ac-AA(1–36)-NH_2	69.2	101.3	8256.38	8257.14
		4	**crude TRI-899**	**59.7**	102.7	4498.17	4498.01
		5	pure TRI-899 NH_4OAc salt	96.0	42.6	4498.17	4497.85
		6	pure TRI-899 TFA salt	N/A[c]	N/A[c]	N/A[c]	N/A[c]
		7	TRI-999	N/A[c]	N/A[c]	N/A[c]	N/A[c]
		overall			**22.8**		
3	Leu_{20} Cut C18-PEG3 incorporated	1A	Ac-AA(1–20)-OH	79.8	N/A[b]	4428.37	4428.48
		1B	Fmoc-AA(21–35)-OH, C18-PEG3 incorporated	94.6	N/A[b]	4267.28	4267.76
		2	H-AA(21–36)-NH_2 C18-PEG3 incorporated	93.1	96.4	4157.31	4157.84
		3	Ac-AA(1–36)-NH_2 C18-PEG3 incorporated	82.5	103.2	8567.66	8567.71
		4	**crude TRI-999**	**66.1**	101.9	4909.50	4909.40
		5	pure TRI-999	97.0	41.1	4909.50	4909.52
		overall			**28.5**[b]		
4	Leu_{16} Cut C18-PEG3 incorporated	1A	Ac-AA(1–16)-OH	83.3	N/A[b]	3679.93	3679.36
		1B	Fmoc-AA(17–35)-OH C18-PEG3 incorporated	90.5	N/A[b]	5015.71	5015.52
		2	H-AA(17–36)-NH_2 C18-PEG3 incorporated	74.9	93.5	4905.74	4906.10
		3	Ac-AA(1–36)-NH_2 C18-PEG3 incorporated	73.6	101.2	8567.66	8567.79
		4	**crude TRI-999**	**58.7**	100.6	4909.50	4909.23
		5	pure TRI-999	97.2	40.6	4909.50	4909.86
		overall			**26.4**[b]		

[a] The reported purity includes 10.1A% of the free side-chain His_{18} species. [b] The synthesis of these fragments started from existing resin-bound fragments, and some of the batches were split during SPPS, so the actual yields could not be calculated accurately. The yields of corresponding fragments from Route 1 SPPS were used to estimate overall yields. [c] Since Route 2 shared the last two stages with Route 1, Route 2 was stopped at Stage 5 and yields of Stage 6 and **7** from Route 1 were used to calculate overall yields.

Chart 1. **Route 1–4 comparison: C18-PEG3 incorporated versus nonincorporated**



was made after observing a 5–15% loss of the trityl protecting group from His_{18} in the N-terminal fragment, Ac-AA(1–20)-OH. It was confirmed that the trityl protecting group was missing from His_{18} by building the fragment using Fmoc-His(Boc)-OH instead of the traditional Fmoc-His(trt)-OH. In the later case, only loss of Boc protecting group was detected in Ac-AA(1–20)-OH. Although no evidence was found to indicate that the loss of this protecting group from His_{18}

negatively impacted the solution assembly in Route 1, the presence of this impurity complicated development of analytical methods and release specifications for this fragment. In an effort to determine whether the trityl was falling off during the solid-phase assembly or during acidic cleavage from the CTC resin, the N-terminal fragment was shortened by four amino acids, which still enabled use of the common Leu-CTC resin. By switching to the disconnect at Leu_{16}, the histidine was pushed near the N-terminal end of the fragment Fmoc-AA(17–35)-OH and was exposed to fewer activation and deprotection cycles.

Routes 3 and 4 on the right side of Scheme 3 represent the C18-PEG3 incorporated strategy. The fragment disconnects followed the same strategy as in the synthesis of TRI-899 at Leu_{20} and Leu_{16}, respectively. The incorporated strategy shares the same N-terminal fragments, Ac-AA(1–20)-OH and Ac-AA(1–16)-OH with TRI-899, while the C-terminal fragments Fmoc-AA(17–30(C18Peg3)-35)-OH and Fmoc-AA(21–30(C18Peg3)-35)-OH contain C18Peg3 instead of Boc protection on Lys_{30}. After global side chain deprotection/decarboxylation and purification, the "incorporated" route yields TRI-999 with no need for further conjugation.

The comparison of this set of routes is summarized in Table 1 and Chart 1. All fragments were synthesized by standard manual SPPS and were completed without the need for recouples or acetylations. The most important data for route comparison are the crude purities after global side chain deprotection/decarboxylation because poor crude purity leads to a difficult, low-concentration, low-throughput chromatographic purification and poor recovery yields. In Routes 1 and 2, the crude peptides are not the final product TRI-999, but TRI-899. However, since the subsequent salt exchange, conjugation, and rework were completed with high purities (>90A%) and yields (mid 80s% to low 90s%), the crude purities of TRI-899 are still good indicators of the route viability for these two routes. The crude purities from Routes 2, 3, and 4 (59.7A%, 66.1A%, and 58.7A%, respectively) are all comparable if not better than that for Route 1 (56.6A%). In Routes 3 and 4, incorporation of C18-PEG3 into the fragments had no affect on the SPPS and subsequent SPS.

The change of fragment disconnect site in Route 2 did indeed reduce the amount of unprotected His_{18} to <1% in fragment Fmoc-AA(17–35)-OH compared to 5–15% in Ac-AA(1–20)-OH in Route 1, indicating that the bulk of the trityl loss occurred during chain elongation, not during resin cleavage. Therefore, moving the histidine residue from the beginning of the N-terminal fragment to the end of the C-terminal fragment could simplify process control. However, this route modification only gave a slight improvement in the purity of the crude TRI-899 (59.7A%, Route 2, vs 56.6 A%, Route 1), indicating the histidine imidazole was relatively unreactive during SPS. There was no observation of lost trityl in Fmoc-AA(17–35)-OH in Route 4 when C18-PEG3 was incorporated into this fragment, supporting the advantage of Leu_{16} as the fragment disconnect site.

Fortunately, efficient HPLC purification of crude TRI-999 was still possible in the presence of the incorporated C18-PEG3 and Routes 3 and 4 have slightly better overall yields (28.5% and 26.4%) than the original routes (18.2% and 22.8%) due to the reduction of process steps. In addition, precipitation of the final product directly after purification instead of lyophilization has been demonstrated on a small scale. The yield advantage, improved efficiency of the process, and potential for a single isolation by precipitation made the "incorporated" routes preferred to the "non-incorporated" routes.

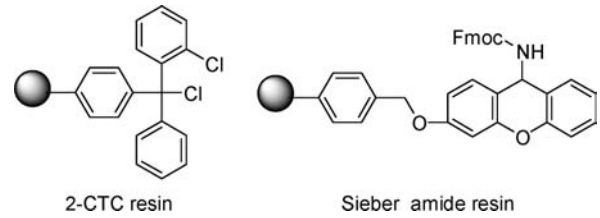


Figure 3. **2-CTC resin and Sieber amide resin.**

Linear and Fragment Routes on Amide Resins

TRI-999, like ENF and T-1249, is acetylated on the N-terminus and amidated on the C-terminus. In the fragment routes based on 2-CTC resin, N-terminal acetylation is performed at the end of the solid phase synthesis, but the amidated amino acid at the C-terminus must be introduced in a separate solution-phase step after cleavage from the CTC resin to a fragment with a free acid at its C-terminus. However, other superacid-sensitive resins, such as Sieber amide resin (Figure 3), are suitable for preparation of fully protected peptide amide fragments. In order to further simplify the TRI-999 synthetic route, the C-terminal fragments were built on Sieber amide resin and cleaved to H-AA(17/21–30(C18Peg3)-36)-NH_2. eliminating one SPS step (Route 5 and 6 in Scheme 4).

Building the C-terminal fragments on Sieber amide resin also allowed the option to build the full length peptide by linear SPPS. For their initial phase I studies, ENF and T-1249 were prepared by linear SPPS on rink amide resin.[18] However, the poor crude purities (30–40A%) and low overall yields (6–8%) from this route were unacceptable for further development. Instead, a solution-phase and solid-phase hybrid convergent process was developed to improve efficiency and throughput.[23] Since our experience with ENF, we have found that peptides similar in size to TRI-999 are typically best assembled via convergent synthesis, and the Route 0.1 synthesis of TRI-899/999 was based on the three-fragment ENF manufacturing route. However, the success in building several 20mers and one 28mer within the sequence of TRI-899 and TRI-999 with acceptable purities, yields, and physical properties suggested that it might be possible to synthesize these peptides linearly. If feasible, a linear synthesis would avoid the isolation of fragments, eliminate the solution-phase assembly, simplify the process, and shorten the cycle time. Therefore, the linear routes (Routes 7 and 8, Scheme 4) were included in route screening.

Again the crude purities of TRI-999 after global side chain deprotection and decarboxylation were the key data to evaluate these new routes. All of the routes based on Sieber amide resin had similar crude purities (66–68A%, Table 2 and Chart 2), comparable to or slightly better than those of Routes 1−4 discussed previously (Scheme 3, Table 1, and Chart 1). Routes

(23) Kang, M.-c.; Bray, B.; Lichty, M.; Mader, C.; Merutka, G. Methods and composition for peptide synthesis (T-20). US 6015881, 20000118, 2000.

Scheme 4. **C18-PEG3 incorporated two-fragment and linear routes based on Sieber amid resin**

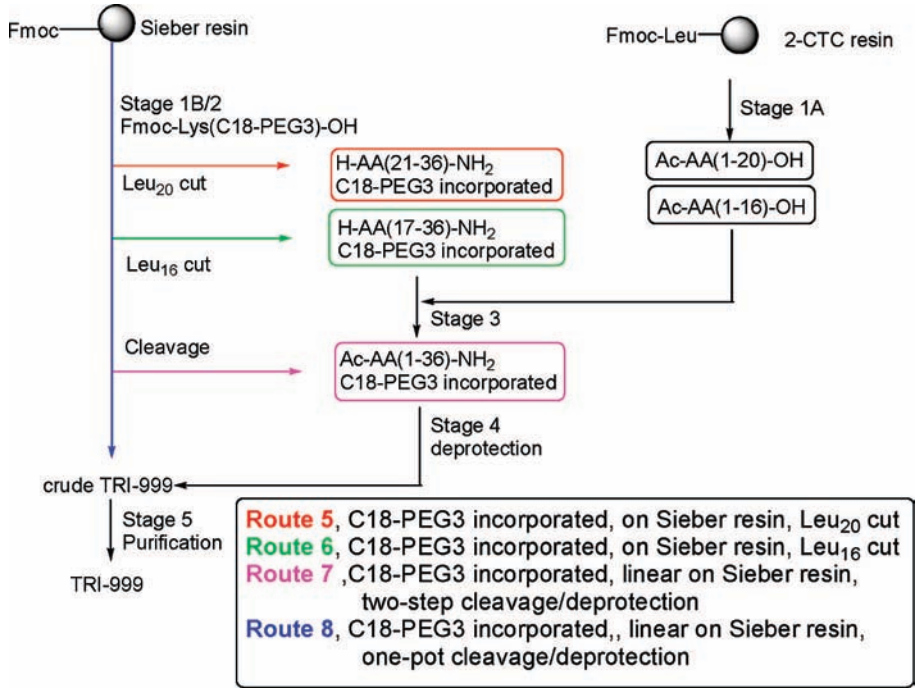


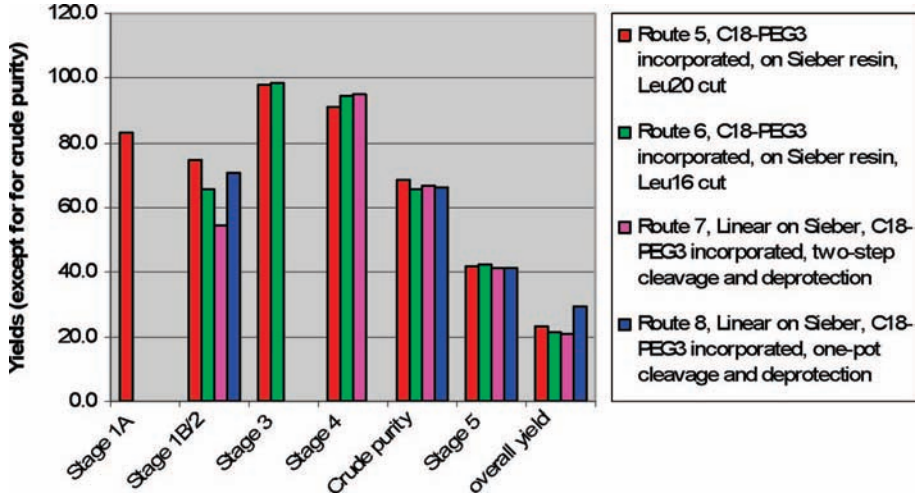
Table 2. **Routes 5–8 comparison: C18-PEG3 incorporated two-fragment and linear routes based on Sieber amide resin**

route	description	stage	description	purity (%)	yield (%)	LCMS (expt)	LCMS (obsd)
5	Leu_{20} Cut C18-PEG3 incorporated, on Sieber	1A	Ac-AA(1–20)-OH	79.8	83.0	4428.37	4428.48
		1B/2	H-AA(21–36)-OH with C18-PEG3 incorporated	93.6	74.7	4157.31	4157.06
		3	Ac-AA(1–36)-NH2 with C18-PEG3 incorporated	72.3	97.9	8567.66	8568.08
		4	**crude TRI-999**	**68.3**	**91.3**	4909.50	4909.04
		5	Pure TRI-999	92.0	41.8	4909.50	4909.40
		overall			**23.2**		
6	Leu_{16} Cut C18-PEG3 incorporated, on Sieber	1A	Ac-AA(1–16)-OH	83.3	N/A	3679.93	3679.36
		1B/2	H-AA(17–36)-NH2 with C18-PEG3 incorporated	88.5	65.4	4905.74	4906.10
		3	Ac-AA(1–36)-NH2 with C18-PEG3 incorporated	65.9	98.3	8567.66	8568.16
		4	**crude TRI-999**	**65.5**	**94.8**	4909.50	4909.06
		5	Pure TRI-999	93.9	42.6	4909.50	4909.23
		overall			**21.5**[a]		
7	linear on Sieber C18-PEG3 incorporated, two-step cleavage and deprotection	1–3	Ac-AA(1–36)-NH2 with C18-PEG3 incorporated	72.0	54.3	8567.66	8567.66
		4	**crude TRI-999**	**66.7**	**95.0**	4909.50	4909.03
		5	Pure TRI-999	92.9	41.1	4909.50	4909.74
		overall			**21.2**		
8	linear on Sieber C18-PEG3 incorporated, one-pot cleavage and deprotection	1–4	**crude TRI-999**	**66.3**	**70.9**	4909.50	4909.24
		5	Pure TRI-999	92.6	41.4	4909.50	4909.13
		overall			**29.4**		

[a] As stated in Table 1, since the actual yield could not be calculated accurately, the recovery yield for stage 1A from Route 1 SPPS was used to estimate overall yield.

5 and 6 are still convergent approaches with C-terminal fragments built on Sieber amide resin, while N-terminal fragments are the same as in Routes 1−4 and were built on 2-CTC resin. Both Routes 7 and 8 are linear syntheses, but the cleavage and deprotection were different. In Route 7, the fully protected peptide was cleaved with 1% TFA (trifluoroacetic acid) in DCM and isolated as Ac-AA(1–36)-NH_2 before the regular global side chain deprotection. Route 8 utilized a one-pot cleavage/deprotection from the resin with TFA/DTT (dithiothreitol)/water (90/5/5). There was no difference in terms of crude purity between these two treatments, but the recovery yield was improved in the one-pot treatment (Route 8) as a result of the stronger acidic condition. In general, the recovery yields from Sieber resin, regardless of fragments or full peptides, were lower than expected, in the range of 60–70%, though the overall yields were in line with the convergent routes because of step reduction in solution-phase assembly. The recovery yields reported here are based on Fmoc loading of the resin reported by the manufacturer, which is determined by

Chart 2. **Routes 5–8 Comparison: two-fragment versus linear synthesis on Sieber resin**



Fmoc-cleavage and can vary depending on the protocols. We prefer to use a loading value based on the amount of the first amino acid attached to the resin. As noticed in the comparison between two-step cleavage/deprotection and one-pot treatment, 1% TFA may not be sufficient to completely cleave the peptide from the Sieber amide resin. Further investigation of this issue is ongoing.

After the initial successful synthesis above, the linear synthesis on Sieber resin (Route 8) was repeated at the scale of 80 g of resin for further evaluation. This build behaved similarly to the small scale trial with crude purity of 66.6A% and recovery yield of 67.3% at crude stage. It is worth noting the increase in the resin bed height during the synthesis. The Sieber resin initial swelling volume in DCM was about 8−9 volumes (as compared to the typical 4–5 volumes for 1% cross-linked 2-CTC resin), and significant growth occurred during peptide chain elongation. By the end of SPPS, the total loaded resin volume almost reached 25 volumes, requiring an increase in the amount of solvent use in order to keep the resin stirrable.

GMP Campaign

Of the numerous routes examined at the time of the start of the first GMP synthesis of TRI-999, the convergent C18-PEG3 incorporated two-fragment route on 2-CTC resin and the linear synthesis of TRI-999 on Sieber resin emerged as the most promising for GMP API production. For this campaign, the higher cost of Sieber resin relative to 2-CTC and the added risk to the project if the linear synthesis failed toward the end of the build were outweighed by the simplification of the process and reduction in the cycle time offered by Route 8. Thus linear synthesis on Sieber resin was chosen for the initial GMP manufacturing of TRI-999. After preparing 1.2 kg of Lys derivative **5**, a campaign was conducted on 1.5 kg of Sieber resin, and the SPPS was successfully completed within 1 month. The fully protected, Sieber-resin-bound TRI-999 was isolated in 76% yield. Though the project was terminated at this stage in favor of alternate NGFI candidate TRI-1144,[15] a sample of crude TRI-999 was cleaved from the resin and isolated in 63% yield (from unloaded resin) with a crude purity of 66A%, demonstrating successful scaling from the previous 80 g run.

Conclusion

This account described the research and development of processes to synthesize TRI-999, a 36 amino acid peptide with C18-PEG3 conjugated on Lys_{30}. The original three- and two-fragment routes were used to produce the parent peptide, TRI-899, which was then converted to TRI-999, with an improved conjugation by using C18-PEG3-OPFP instead of in situ activation. Further advances were made by the incorporation of C18-PEG3 into the solid-phase synthesis via a novel Fmoc-protected amino acid, Fmoc-Lys(C18-PEG3)-OH. Several two-fragment "incorporated" routes and the linear synthesis of TRI-999 were examined, which further simplified and shortened the process. Successful production of TRI-999 via linear synthesis was demonstrated in a kilogram-scale GMP campaign on Sieber amide resin.

Experimental Section

General. All incorporated raw materials were purchased from commercial sources and for the GMP campaign were qualified against internal specifications prior to use. All non-incorporated raw materials were accepted upon the vendors' COAs and used without further purification. Analytical HPLC was performed on an Agilent 1100 system (Agilent) with UV detector and/or PL-ELS 1000 detector (Polymer Laboratories). Semipreparative HPLC was performed on a ProStar 210 system (Varian). Preparative HPLC was performed on a DYNAMAX SD-1 system (Varian) with slurry pack of Kromosil C8-10-100 silica (Eka Chemicals AB) in a 15-cm column (DYNAMAX RAMPAK, Varian). LC-MS was performed on an LCQ ESI-IT mass spectrometer (Thermo Finnigan) and Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems).

C18-PEG3-PFP (3). To a 25-L reactor was charged C18-PEG3-OH (**2**)[21] (778 g, 1.811 mmol, 1 equiv) and pentafluorophenol (334 g, 1 equiv). These were dissolved in DCM (7.78 L, 10 vol), and the resulting solution was cooled to 0–5 °C. Diisopropylcarbodiimide (284 mL, 1 equiv) was added, and the solution was warmed to 25 ± 5 °C. The suspension was stirred for about 2 h, and the reaction was shown to be complete by HPLC. The mixture was cooled, filtered, and washed with DCM (2 vol, 1.56 L) to remove diisopropylurea. The filtrate was concentrated to remove most of the solvent. Heptane (7.78

L, 10 vol) was added, and the distillation was continued to remove the remaining DCM. The resulting suspension was cooled to 20–25 °C, and the precipitate was isolated by filtration, washed with heptane (2 × 2 vol, 1.56 L each) and dried at 35 ± 5 °C under N_2 bleed to constant weight, yielding 1074 g of **3** (99.5%, 100.0A% HPLC with ELSD and UV detection, LC-MS 595.30 Da, exp. MW 595.33 Da).

Fmoc-Lys(C18-PEG3)-OH (5). To a 25 L Reactor was charged Fmoc-Lys-OH•HCl (747 g, 1.85 mol, 1.03 eq), DMF (1067 mL, 10 vol to C18-PEG3-PFP), and DIEA (321 mL, 1.85 mol, 1 eq) and the resulting solution was stirred at ambient temperature for 5 min. C18-PEG3-PFP (**3**) (1067 g, 1.79 mol, 1 eq) was added and the solution was stirred at 20–25 °C until complete by HPLC w/ ELSD detection. Then the solution was cooled to 0–5 °C and the product was precipitated by the addition of prechilled 0.01N HCl (14.9 L, 14 vol) while keeping the temperature below 15 °C. The resulting slurry was stirred at 15–20 °C for 30 min and the product was isolated by filtration, washed with water (4 vol, 4.27 L), and dried until the solid could be easily removed. The solid was placed into trays and drying was completed under vacuum with N_2 bleed at 35 ± 5 °C. The solids were reslurried in MTBE (10 vol, 10.7 L) for 2 h at 20–25 °C and then reisolated by filtration, washed with MTBE (8 vol, 8.5 L), and dried as before to yield 1165 g **5** (83.4%, 97.1A%, LC-MS 779.50 Da, exp. MW 779.51 Da).

Genearal Procedure for SPPS. Peptides were synthesized by solid-phase peptide synthesis (SPPS) using classical fluorenylmethyloxycarbonyl (Fmoc) protection methods.[24] The following side chain protecting groups were used: *tert*-butyl ether (tBu) for tyrosine and threonine; *tert*-butyl (OtBu) ester for glutamic acid and aspartic acid; trityl (trt) for glutamine and asparagine; *tert*-butoxycarbonyl (Boc) for lysine and tryptophan, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine. The 2-CTC resin was swollen in DCM (10 vol) for 15–30 min and then drained while a solution of Fmoc-AA-OH (0.45 equiv) and DIEA (1.35 equiv) was prepared in DCM (5 vol). The resin was reslurried in DCM (5 vol) and drained. The amino acid solution was added to the resin, and the slurry was agitated for 2 h. The vessel was drained, and the resin was slurried in NMP (4 vol). The NMP was drained, and the resin was end-capped with 9:1 (v/v) MeOH/DIEA (6 vol) for 45 min. The vessel was drained, and the resin was washed with DCM (6 vol × 2). A sample of resin was removed for loading level determination. The resin was then washed with NMP (4 vol) and stored while the loading level was determined.

N-Terminal Fmoc groups were removed by two consecutive 20–30 min treatments with 10% piperidine in NMP followed by washes with NMP to a negative chloranil test.[25] Amino acid couplings were performed by preactivating the side chain protected Fmoc amino acid (1.5 equiv) with 6-Cl HOBt (1.5 equiv) and DIEA (1.7 equiv) in DMF and cooling to 0–5 °C. TBTU (1.5 equiv) was added, and the solution was stirred at 0–5 °C for 15 min prior to addition to the resin. The reaction was monitored to completion by the Kaiser test.[26] A recouple with 0.5 equiv was performed after 3 h if necessary. For N-terminal acetylated fragments, the acetylation was completed by treatment with pyridine (6 equiv) and acetic anhydride (5 equiv) in NMP until a negative Kaiser test was obtained. After completion of a fragment, the resin was washed with NMP (5×) and DCM (5×) and then cleaved with 1% TFA/DCM for 45 min at 0–5 °C. The TFA was quenched with pyridine (1.26 vol equiv), and the resin was washed with DCM until no more product was detected. The DCM was removed under vacuum and replaced with a small portion of IPA. The solution was cooled to 0–5 °C, and the product was precipitated with water, filtered, washed, and dried in a vacuum oven. Characterization was performed by HPLC and LC-MS (Tables 1 and 2).

Genearal Procedure for SPS. The reactor was charged with DMF (10 vol) followed by fragment acid (1 equiv), fragment base (1.05 equiv), 6-Cl HOBt (1.3 equiv) and DIEA (1.5 equiv). On larger scale, the equivalency was determined by use test. The mixture was stirred to dissolve all solids and then cooled to 0–5 °C. TBTU (1.2 equiv) was added, and the reaction mixture was stirred at 0–5 °C for 10 min. The solution was warmed up to 25 °C and stirred until the reaction was complete by HPLC. If removal of Fmoc protecting group was needed, piperidine (4 equiv) was added, and the reaction was stirred at 25 °C until the deprotection was complete by HPLC. The solution was cooled to 0–5 °C, and precooled water (<5 °C, 10 vol) was added slowly while maintaining a temperature below 10 °C. The suspension was stirred at below 10 °C for 30 min and then filtered. The solid was washed with 20% EtOH/water (4 vol × 2) and dried. The damp solid was reslurried in 20% EtOH/water at 25 °C for 3 h, then filtered, washed with 20% EtOH/water (5 vol), and dried. Characterization was performed by HPLC and LCMS (Tables 1 and 2).

General Procedure for Side Chain Deprotection. A solution of TFA/DTT/water (10 vol, 90/5/5) was prepared and cooled to below 10 °C. The fully protected peptide was added while maintaining a temperature below 15 °C. The mixture was heated to 20 °C, and the resulting solution was stirred for up to 5 h. During this time MTBE (25 vol) was cooled to 0–5 °C. The peptide solution was cooled to 0–5 °C, and the MTBE was slowly added. The slurry was stirred for 30 min, and the solids were collected by filtration and washed with MTBE (5 vol × 3). The product was dried on the filter until it could be easily removed and then returned to the reactor, which had previously been charged with acetonitrile (10 vol), acetic acid (0.1 vol) and DIEA (0.1 vol). The pH was adjusted if necessary with DIEA and/or acetic acid to the desired range of 4–5. The slurry was stirred at 20–25 °C until HPLC showed no change in the product profile. The solids were isolated by filtration, washed with acetonitrile (5 vol × 3,) and dried to give the crude peptide. Characterization was performed by HPLC and LCMS (Tables 1 and 2).

General Procedure for Purification. Crude peptide was purified on Kromosil C8 RP silica in basic buffer system (50 mM ammonium acetate, NH_4OAc, in water, pH 8.5 ± 0.1 as Buffer A and acetonitrile as Buffer B). The column was washed

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with at least one column volume (CV) of 95% Buffer B and then equilibrated in 25% Buffer B for at least 3 CV. The crude peptide was dissolved in 25% Buffer B at a concentration of 35 mg/mL and then filtered through 0.45 μm microfiber filter. The clear solution was pumped onto the column and the flask, and the line was washed with 25% Buffer B. The purification was performed using the linear gradient from 28% to 33% Buffer B in 40 min for TRI-899 or from 33% to 38% Buffer B in 40 min for TRI-999. Acceptable fractions by HPLC were pooled and lyophilized to yield pure peptides.

The salt exchange of TRI-899 was performed on Kromosil C8 RP silica in TFA buffer system (0.1% TFA in water as Buffer A and 0.1% TFA in acetonitrile as Buffer B). The column was washed with at least 1 CV of 95% Buffer B and then equilibrated in 20% Buffer B for at least 3 CV. TRI-899 NH_4OAc salt was dissolved in 20% acetonitrile at a concentration of 25 mg/mL, pH 8, and then filtered through 0.45 μm microfiber filter. The clear solution was pumped onto the column and the flask, and the line was washed with 20% Buffer B. The column was washed by 20% B for at least 4 CV and then 70% B for 3 CV. Fractions were pooled, diluted and lyophilized to yield pure TRI-899 TFA salt.

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