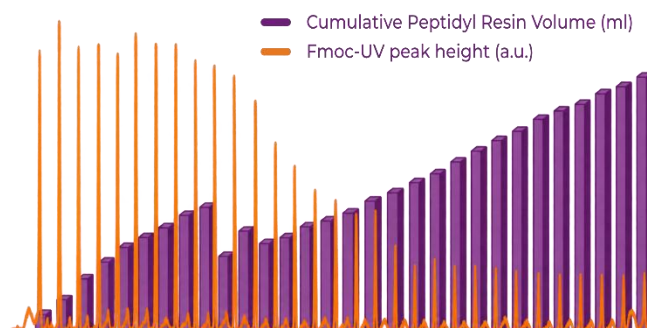


Application Note 83:

Using Real-Time Aggregation Monitoring to Optimize the FF-SPPS of Barstar (61–90)



Abstract

This application note demonstrates how sequence-based aggregation prediction and real-time VBFR™ resin-volume data from the Peptide-Builder™ can be combined to guide method optimization in Fast-Flow solid-phase peptide synthesis.

Using the challenging Barstar fragment (61–90), predicted aggregation-prone regions were compared with VBFR data to identify where peptide-chain growth was disrupted.

Five aggregation-prevention strategies were then evaluated, including Thr(Trt), an ArgTag, a pseudoproline and two combined approaches.

Individual strategies reduced or displaced the observed aggregation, while combining the ArgTag with Fmoc-Thr(Trt)-OH eliminated the observed aggregation signatures from the VBFR profile. The study illustrates how predictive analysis and in-process data can support the development of sequence-specific synthesis strategies.

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Background

Peptide aggregation during SPPS [1] remains one of the major challenges faced by synthetic chemists when optimizing difficult sequences.

Diagnostic tools such as Peptalyzer™ use sequence-based models incorporating topological mapping and biophysical parameters to identify regions with an increased propensity for aggregation [2, 4]. These predictions can help guide the initial synthesis strategy. However, predictive models cannot determine [3] how a particular sequence will behave under specific synthesis conditions, and interventions intended to disrupt one aggregation-prone region may alter peptide conformation and reveal aggregation elsewhere in the growing sequence.

If we take for example the Barstar fragment (61-90), software analysis shows that T86-I85 is a beta-sheet prone region of the peptide with a high risk of aggregation during its synthesis.[4]

Figure 1, shows a traffic light style chart highlighting the problematic synthetic steps, and this specific region seems to be of special consideration as it is located way too early in the synthetic process. Because this region occurs early in the synthesis, with approximately 50 synthetic steps remaining, disruption of peptide-chain growth at this stage has the potential to affect the efficiency of subsequent coupling and deprotection steps.

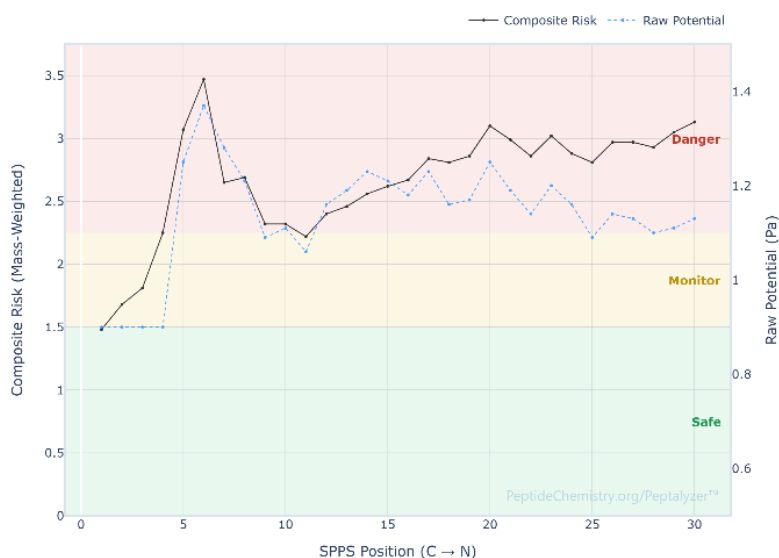


Figure 1 - Aggregation prediction for Barstar

Peptalyzer can also suggest strategies to prevent this. In this case, due to the presence of a Threonine residue, the use of a bulkier protecting group or a pseudoproline approach could benefit the peptidyl chain growth.

Now, this is not the only point the analysis brings attention to, as A78 and S70 are two potentially problematic steps and analysis of the sequence with the introduction of the prevention strategies is not possible as it is only capable of scrutinising the native composition of the desired peptide and ignores modifications on the amino acids used during Fmoc/tBu SPPS.

Our previous application note demonstrated how VBFR resin-volume monitoring can identify aggregation signatures during synthesis. Here, we use this information to evaluate strategies for reducing aggregation in Barstar (61-90).

Experimental Conditions

Parameter	Condition
Target peptide	Barstar (61-90): H-KQLTENGAE ^{SVLQVF} REAKAEGCDITIILS-NH ₂
Resin	TentaGel S Rink Amide resin
Resin loading	0.24 mmol/g
Synthesis scale	0.100 mmol; 0.42 g of resin
Synthesis platform	Peptide-Builder™ with Variable Bed Flow Reactor (VBFR™)
Solvent	DMF
Activation chemistry	DIC/Oxyma activation of Fmoc-protected amino acids
Reagent equivalents	Fmoc-protected amino acid: 5 equiv.; Oxyma: 7.5 equiv.; DIC: 7.5 equiv., relative to resin-bound reactive sites
Fmoc deprotection	20% (v/v) piperidine in DMF, without additives
Coupling temperature	80 °C; arginine couplings at 60 °C
Fmoc-deprotection temperature	80 °C
Post-coupling wash	4.5 mL DMF
Post-deprotection wash	5.0 mL DMF
Strategy specific changes	Thr(Trt) substitution; ArgTag assembly; pseudoproline incorporation; corresponding combinations

All syntheses used the conditions listed above, except for the strategy-specific changes described in results and discussion.

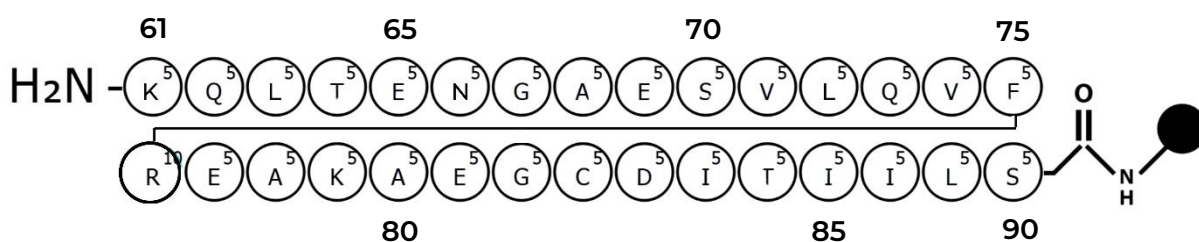
Resin-bed volume was monitored using the VBFR™ and calculated from the displacement of the upper plunger. Cumulative resin-volume change is reported in mL relative to the volume at the start of the first Barstar coupling, corresponding to the C-terminal serine (Ser90). For syntheses without ArgTag, this reference was established before the first coupling. For syntheses with ArgTag, it was established after assembly of the six arginine residues, immediately before coupling Ser90. The cumulative volume change was therefore set to zero at the start of Barstar synthesis in each experiment, and the plotted profiles exclude the initial ArgTag assembly.

Results and Discussion

To demonstrate the usefulness of the VBFR real time analysis as a tool to introduce into your method development workflow we decided to evaluate the potential aggregation disruption abilities of different strategies based on modifications that affect the backbone of the peptide chain growth. Either by disrupting hydrogen bond formation, structural constriction or promotion of α -helical secondary structures of the resin bound peptidyl chain, the tools we have access currently need to be evaluated to find the best option available.

Standard SPPS synthesis:

We used the Barstar fragment (61-90) as our model problematic sequence as we have previously worked with it and found a specific step that was causing the main aggregation event. Although the cumulative chart analysis shows a severe aggregation event during the 7th cycle (D84), the in-depth analysis of the VBFR volume shows that the deviation begins on the previous coupling step (I85) and it extends for the next three steps. (Figure 2)



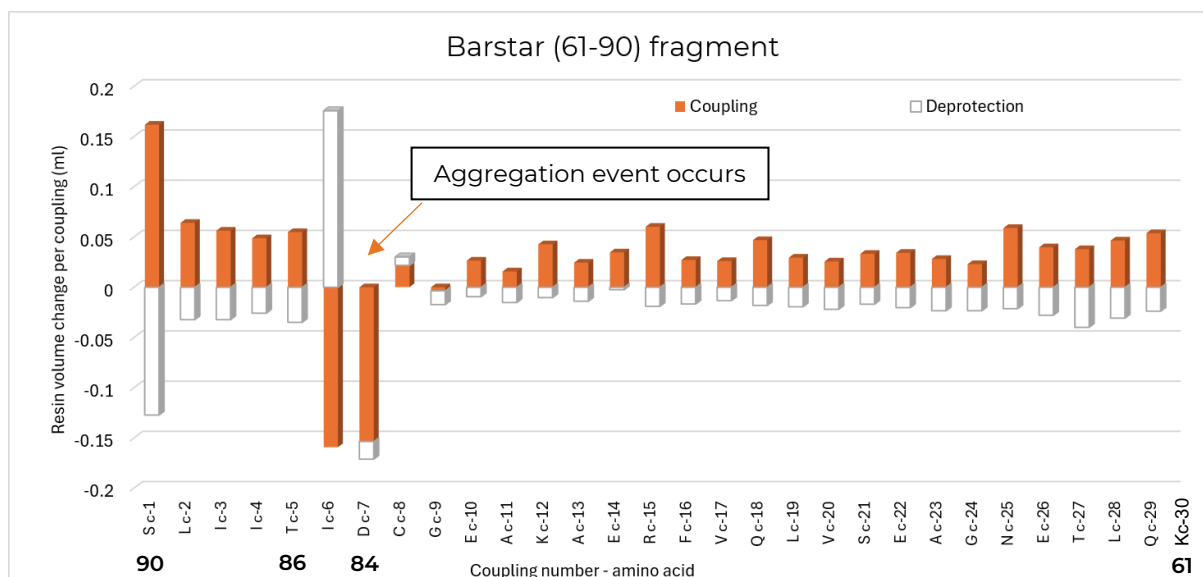
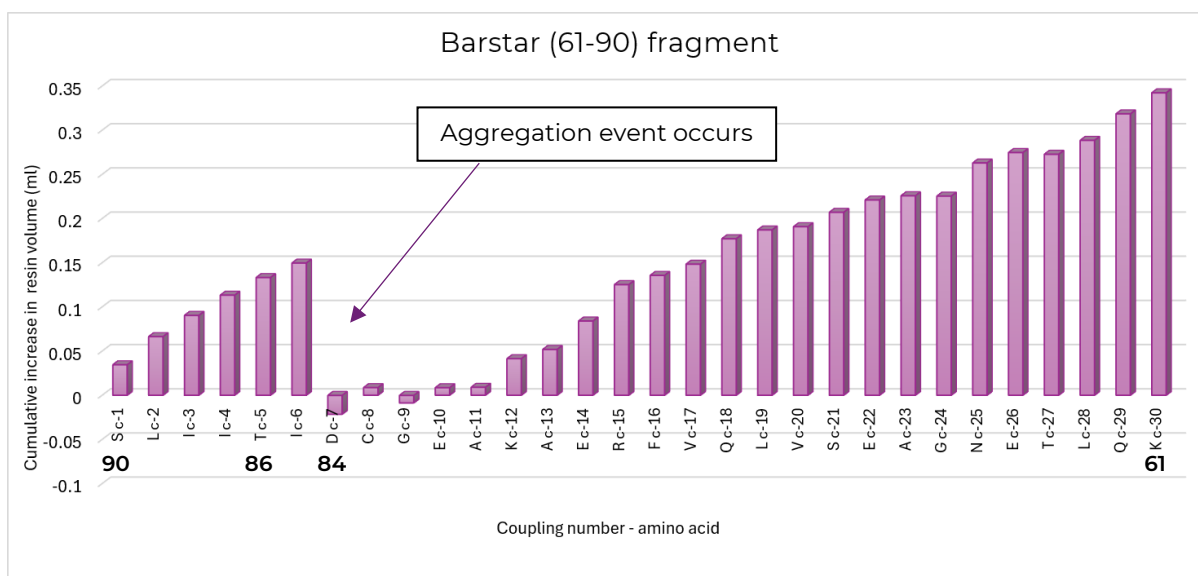


Figure 2 - Cumulative and detailed aggregation chart for Barstar using fragment (61-90)

The experimentally observed VBFR response is consistent with the aggregation-prone region predicted by Peptalyzer, which identified the region around T86–I85 as having an increased aggregation risk.

Sterically hindered protecting groups:

Fmoc-Thr(Trt)-OH provides a bulkier side-chain protecting group than the more commonly used Fmoc-Thr(tBu)-OH. The increased steric bulk can disrupt hydrogen bonding and inter-chain association associated with β -sheet formation and peptide aggregation.

Although the cumulative resin-growth profile improved compared with the standard synthesis using Fmoc-Thr(tBu)-OH, the VBFR data indicate that the aggregation event continued to influence approximately three subsequent coupling cycles as Figure 3 shows.

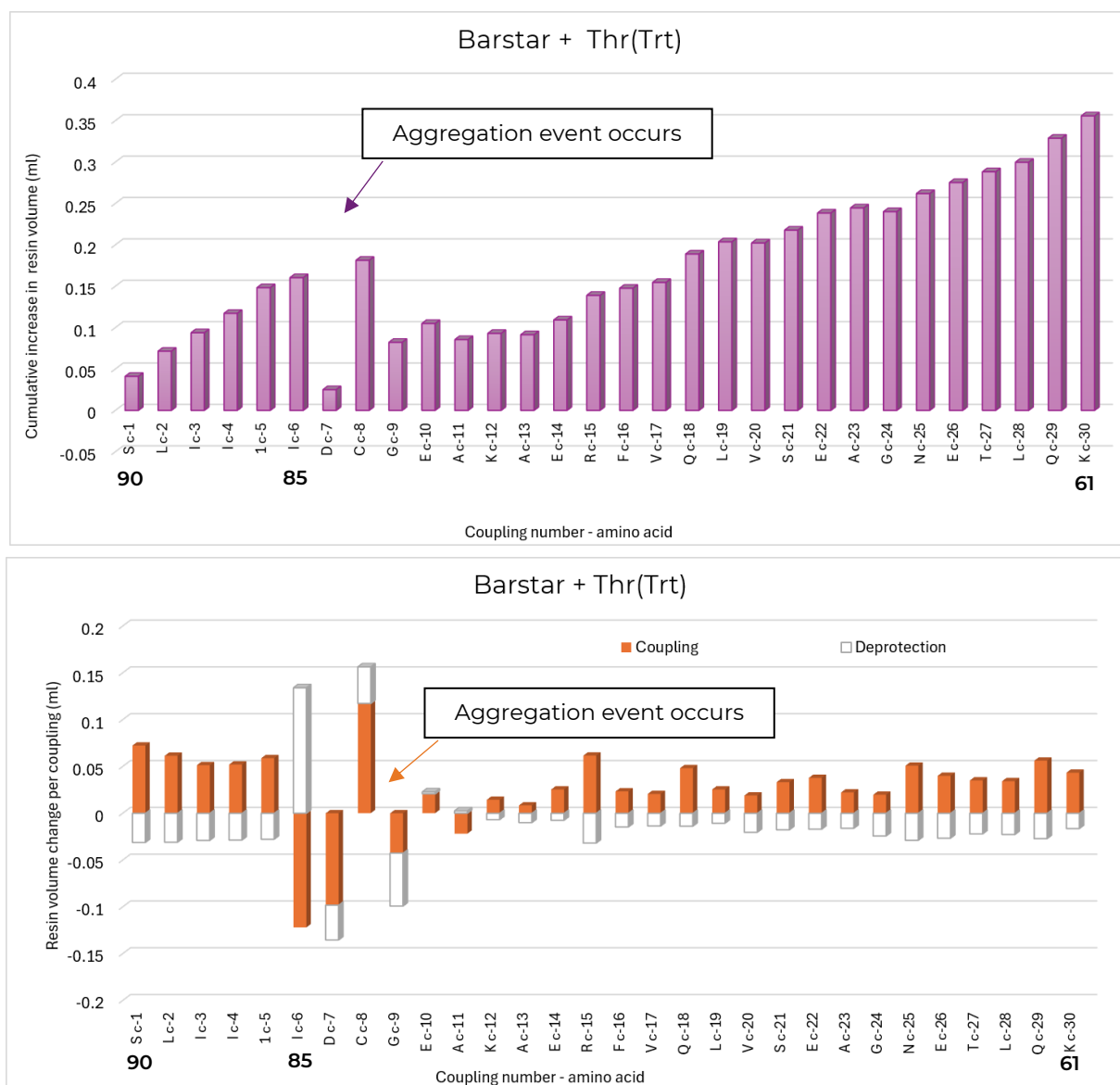


Figure 3 - Cumulative and detailed aggregation chart for Barstar using Fmoc-Thr(Trt)-OH (I)

ArgTag:

Our next implemented strategy was reported not long ago by the group of Prof. Hartrampf [5]. An ArgTag comprising six arginine residues was assembled at the C-terminus before synthesis of the Barstar sequence. Introduction of the ArgTag suppressed the original aggregation signature around I85. However, continued monitoring revealed a second aggregation event around E81–A80. Examination of the individual VBFR responses indicated that the disturbances were associated primarily with coupling rather than deprotection steps in both tagged and untagged sequences (Figure 4). The ArgTag was not removed in this study.

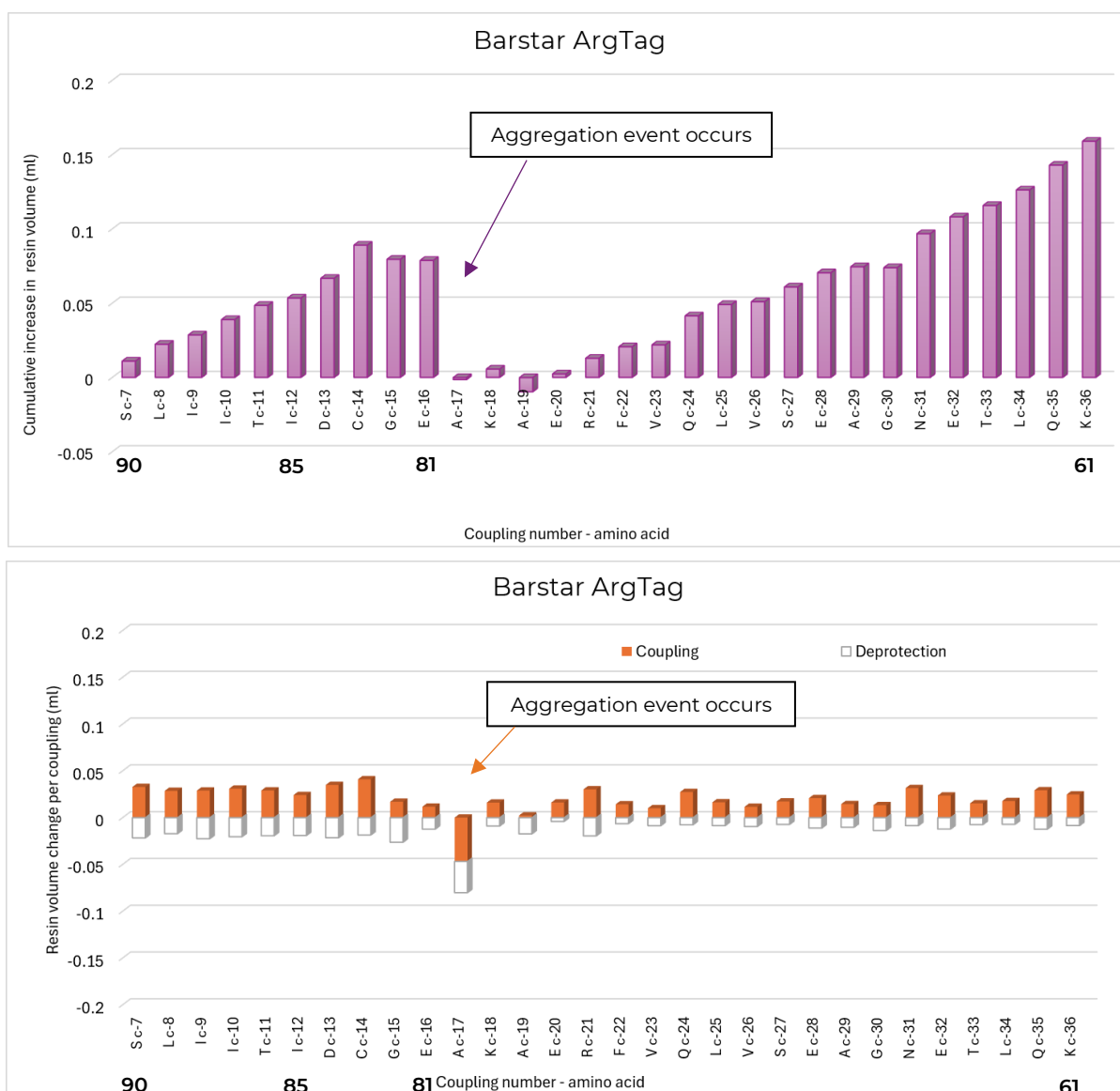


Figure 4 - Cumulative and detailed aggregation chart for Barstar using ArgTag

Pseudoprolines:

The next strategy evaluated was the use of a pseudoproline dipeptide, as suggested by the sequence-based analysis. Pseudoprolines are commonly used in SPPS to disrupt the formation of secondary structures that can promote peptide aggregation. Incorporation of a pseudoproline introduces a reversible conformational constraint into the growing peptide backbone [6,7], disrupting inter-chain hydrogen bonding and reducing the tendency to form β -sheet-like structures.

Pseudoproline building blocks can be introduced at suitable Ser, Thr or Cys residues, where the side-chain heteroatom enables formation of a five-membered oxazolidine or thiazolidine ring incorporating the backbone nitrogen to form a locked dipeptide unit with the preceding amino acid. They are typically supplied as protected dipeptide building blocks and can be incorporated using conventional Fmoc SPPS coupling conditions.

For the Barstar (61–90) sequence, the predicted aggregation-prone region around Thr86–Ile85 provided a suitable position for this approach. Fmoc-Ile-Thr(ψ Me,Me-Pro)-OH was therefore introduced during the fifth coupling step of the synthesis. The resulting VBFR profile showed that the aggregation behaviour previously observed around Ile85 was suppressed.

The pseudoproline successfully suppressed the aggregation behaviour previously observed around I85. However, real-time monitoring revealed a later aggregation event around S70–E69. This result demonstrates an important limitation of addressing an individual predicted aggregation site: disrupting aggregation in one region can expose aggregation behaviour later in the growing peptide chain. (Figure 5)

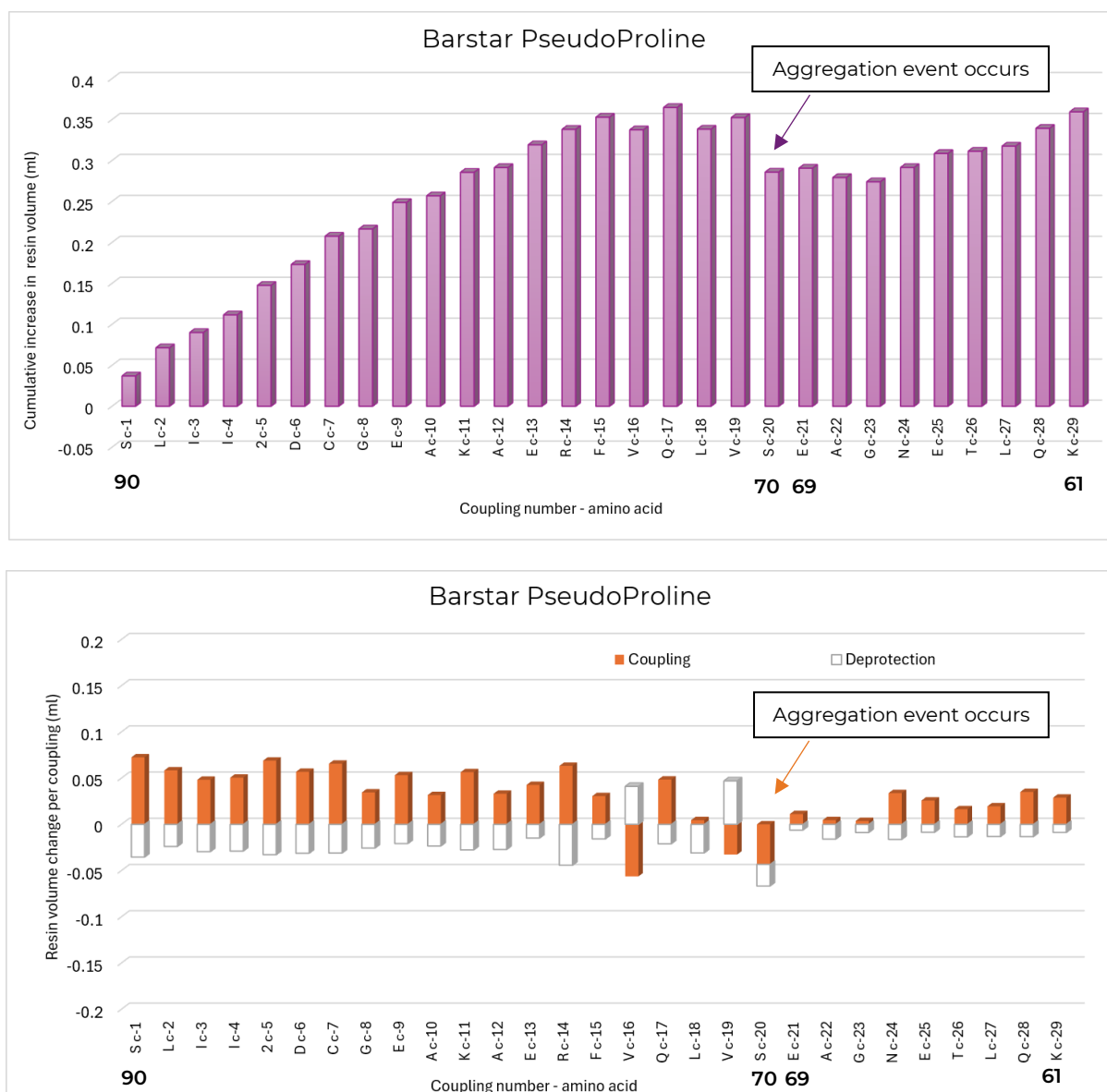


Figure 5 - Cumulative and detailed aggregation chart for Barstar using Fmoc-Ile-Thr(ψ Me,Me-Pro)-OH (2)

Combined strategies:

As our final attempts we decided to combine strategies to see if we could prevent all aggregation effects. Our logical way of moving forward was using both strategies that could be implemented on the same synthesis. Combining the ArgTag with the pseudoproline building block produced a VBFR profile similar to that observed with the pseudoproline alone. The later aggregation region remained apparent, indicating no clear additional benefit from combining these two strategies (Figure 6).

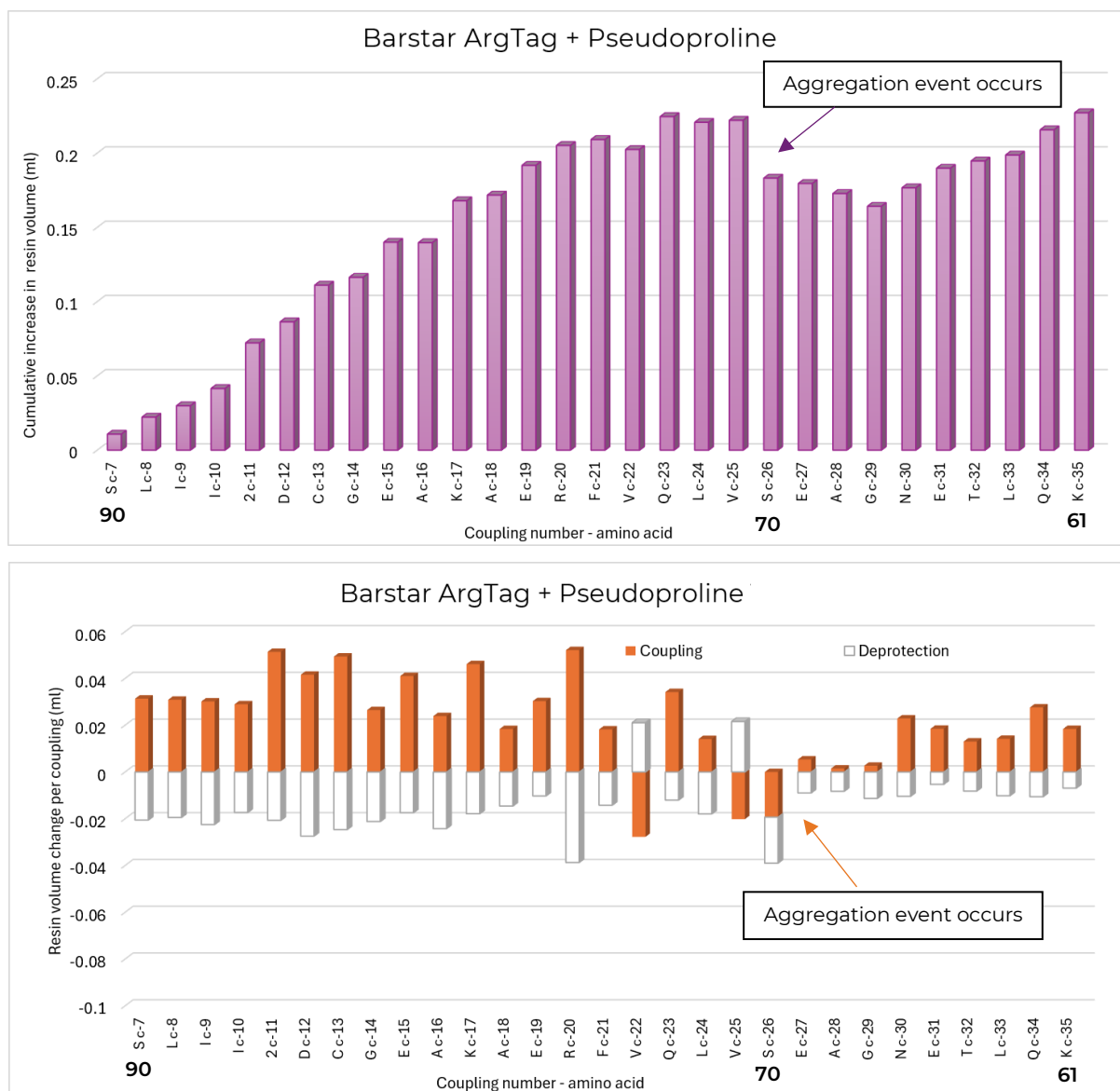


Figure 6 - Cumulative and detailed aggregation chart for Barstar using ArgTag and Fmoc-Ile-Thr(ψMe,Me-Pro)-OH

In contrast, combining the ArgTag with Fmoc-Thr(Trt)-OH produced a substantially more consistent resin-growth profile. No equivalent aggregation signatures were observed in the VBFR data over the remainder of the synthesis (Figure 7).

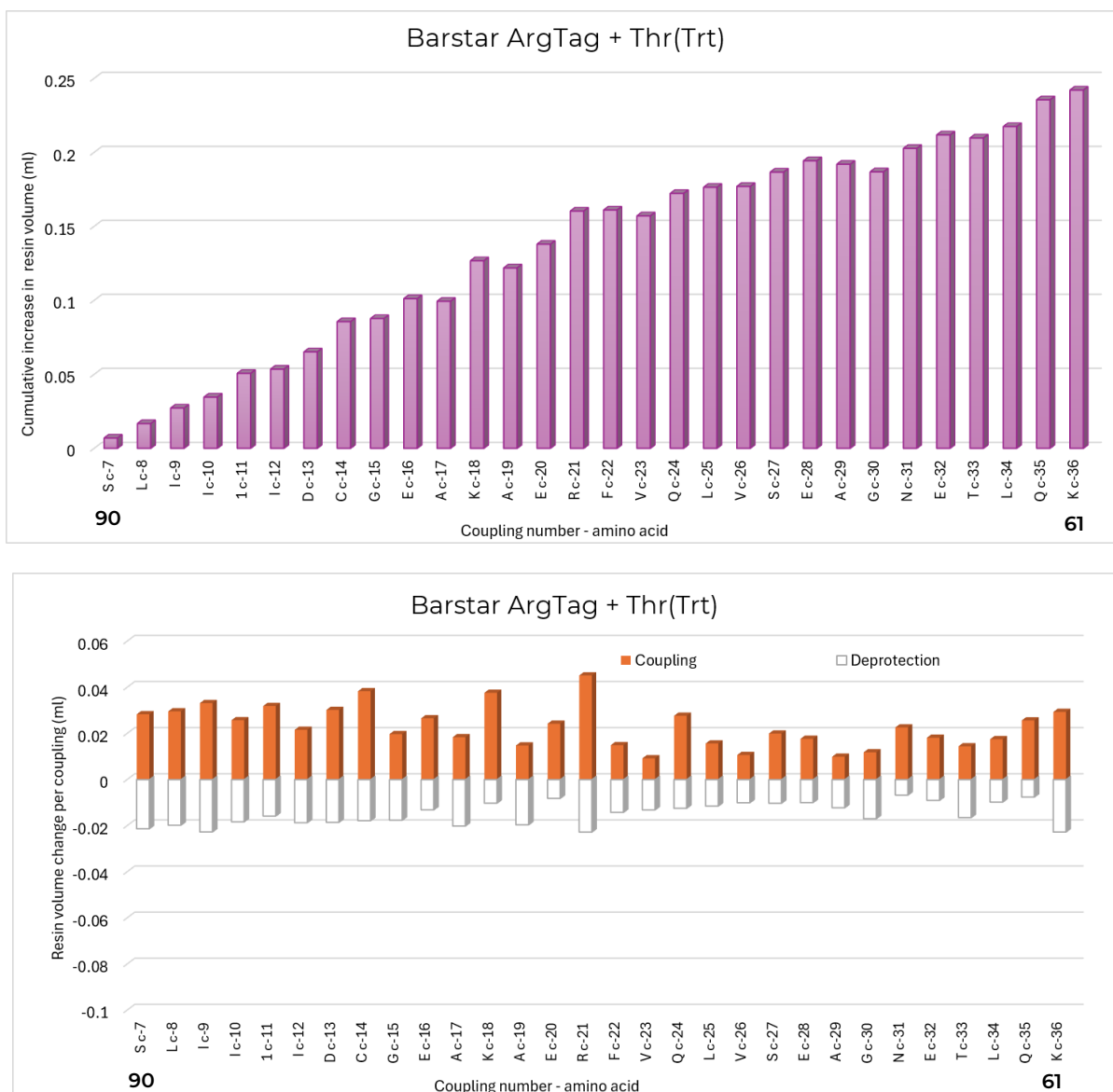


Figure 7 - Cumulative and detailed aggregation chart for Barstar using ArgTag and Fmoc-Thr(Trt)-OH (1)

Conclusions

Five strategies for mitigating aggregation during the FF-SPPS of Barstar (61–90) were evaluated using real-time VBFR resin-volume monitoring. Each intervention produced a distinct resin-growth profile, demonstrating that suppression of an initial aggregation event does not necessarily prevent aggregation later in the synthesis.

Of the five strategies evaluated and summarised in Table 1, the combination of ArgTag and Fmoc-Thr(Trt)-OH produced the most consistent resin-growth profile, with no equivalent aggregation signatures observed during the remainder of the synthesis.

Strategy	Effect at initial aggregation region	Later aggregation	Overall VBFR interpretation
Standard synthesis	Strong disruption beginning at I85	Further effects over subsequent couplings	Baseline aggregation profile
Thr(Trt)	Reduced severity	Effect continued for approximately three couplings	Partial improvement
ArgTag	Prevented the initial event	Secondary event around E81–A80	Aggregation displaced / delayed
Pseudoproline	Prevented the initial event	Later event around S70–E69	Initial aggregation prevented; later aggregation observed
ArgTag and pseudoproline	Similar to pseudoproline alone	Later aggregation remained	No clear additional benefit over the pseudoproline on its own
ArgTag and Thr(Trt)	No equivalent disruption observed	No further aggregation signature observed	Best VBFR profile from interventions tested

Together, these results demonstrate how real-time VBFR monitoring can support a more systematic approach to method development for difficult peptide sequences. Rather than relying solely on end-point analysis, changes in resin-bed behaviour can be observed as the peptide chain grows, allowing the effect of an intervention to be assessed and subsequent problematic regions to be identified during synthesis.

The integration of VBFR monitoring within the Peptide-Builder™ therefore provides experimental information that can complement sequence-based prediction and support the development of sequence-specific synthesis strategies.

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