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Control of aspartate epimerization during the coupling of caspase specific tetrapeptides with aromatic amines by using *N*-[[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]-pyridin-1-yl]methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) as a coupling reagent

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Abstract—During the synthesis of new caspase substrates, we have encountered extensive aspartate epimerization upon coupling of *t*-Bu-protected tetrapeptides Ac-IETD-OH or Ac-DMQD-OH with aromatic amines (aminocoumarins and aminoquinolines) by using aminium-based coupling reagent HATU in the presence of 2,4,6-trimethylpyridine (TMP). To study this reaction in more detail, an RP-HPLC method was developed that afforded the separation of the epimers. By carefully adjusting the reaction conditions, the epimerization could be reduced to very small levels (from 75% down to below 3%). A new, highly hindered base 2,4,6-tri-*tert*-butyl-pyridine (TBP), was found to be superior to the traditional TMP in the catalysis of this reaction. Moreover, the aspartate epimers were found to be interconvertable at elevated temperatures in an inert solvent, whereby the D-aspartate containing epimer was the thermodynamically controlled product. Over the course of the study, new side products of HATU coupling reactions, the *N*,*N*-dimethylamides of the aspartic residue of above tetrapeptides, were also identified.

1. Introduction

High enantiomeric purity and methods for preventing epimerization are essential for successful peptide synthesis since the presence of diastereomeric impurities can considerably lower the yield and the purity of the products, thus lowering the biological activity of the products.[1] It is now recognized that the structural parameters influencing epimerization are quite complex and that the degree of epimerization is often influenced by the structure of the amino acid,[2] the type of solvents,[3] the nature of the group attached to the N-terminal end of the amino acid,[3] the coupling reagent,[4] and the organic used base.[5] The loss of configuration occurs almost exclusively under the basic conditions during activation of the carboxy group and during the coupling reaction.

Synthetic progress over the last four decades has resulted in a variety of new techniques and coupling reagents, which allow for the synthesis of products in high yield and high enantiomeric purity. Among them are the aminium- and phosphonium-based coupling reagents,[6,7] such as HATU or benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP), respectively. HATU has become one of the most popular coupling reagents in solid phase as well as in solution phase peptide synthesis. However, the precise mechanism of action of this compound is still unknown. It is believed (Scheme 1) that the highly

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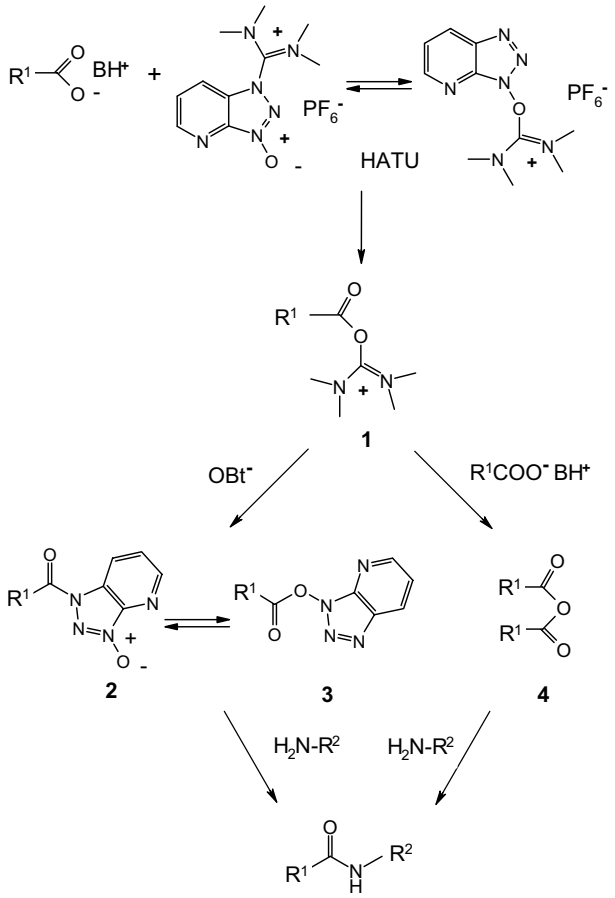
reactive intermediate **1**[8,9] is involved in the formation of active ester **3**, which was found to play a major role in this coupling.[10,11] Depending on the conditions of the reaction, **3** exists as a simple ester or in equilibrium with the corresponding *N*-oxide **2**, which has been isolated.[11,12] However, an alternative pathway via a symmetrical anhydride **4**[13] cannot be excluded.



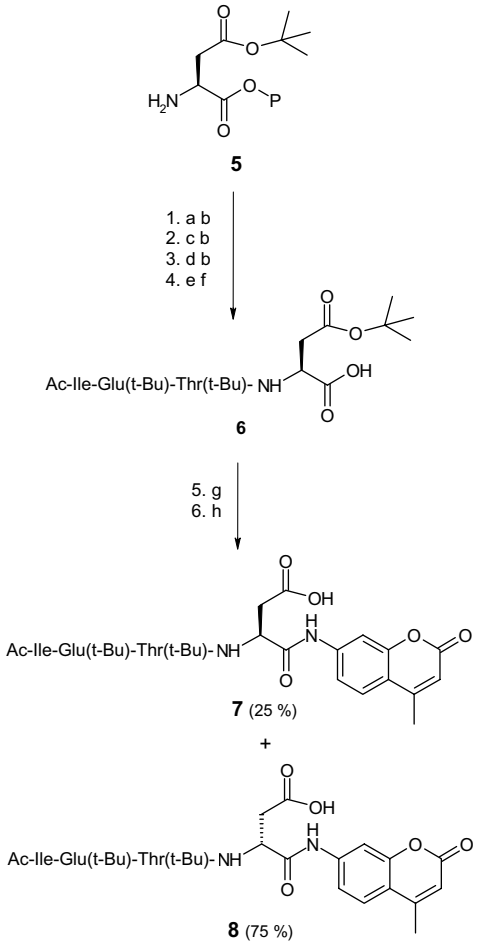
Scheme 1. Mechanism of HATU mediated peptide bond formation.

In our efforts to develop novel caspase substrates for the detection of apoptosis in cancer cells, the synthesis of new caspase-8 substrates was undertaken. Our synthetic strategy was to couple a *t*-Bu-protected caspase-8 specific tetrapeptide Ac-Ile-Glu(*t*-Bu)-Thr(*t*-Bu)-Asp(*t*-Bu)-OH by the free carboxylic acid with a series of new fluorescent aromatic amines. For this purpose, the widely used coupling reagent HATU in the presence of TMP in DMF was used. To optimize the reaction conditions, we first coupled the well known 7-amino-4-methyl-coumarin (AMC) and compared the deprotected product with the commercially available one. However, the RP-HPLC, CD and NMR analyses of the product revealed that the aspartic acid residue had specifically epimerized to give up to 75% of the D-diastereomeric Ac-Ile-Glu-Thr-D-Asp-AMC. The same effect was observed when other aromatic amines were used. This particular coupling reaction proved to be an excellent model system for studying epimerization by RP-HPLC in general. By carefully optimizing the reaction conditions, we have now obtained the desired products in >97% enantiomeric purity and without significant reductions in yield. Our new method is applicable to the coupling of a variety of aromatic amines, as well as to other caspase relevant tetrapeptide sequences. Furthermore, we herein report that TBP is superior to TMP in this particular coupling sequence. Finally, a new side product formed in HATU coupling reactions with DMF as a solvent is described here in detail for the first time.

2. Results and discussion

2.1. Epimerization of aspartic acid in the synthesis of Ac-IETD-AMC

The chemical route providing the desired caspase-8 Ac-IETD-AMC substrate is depicted in Scheme 2. It was decided to first synthesize the protected tetrapeptide, which was common to all the products needed in our synthesis, and subsequently couple with different fluorescent aromatic amines. Tetrapeptide **6** was synthesized by a standard Fmoc solid-phase procedure by using 2-chlorotrityl re-



Scheme 2. Synthesis of Ac-IETD-AMC (**P**: 2-Chlorotrityl resin (a) Fmoc-Thr(*t*-Bu)-OH/HATU/TMP; (b) 20% Piperidine/DMF; (c) Fmoc-Glu-(*t*-Bu)-OH/HATU/TMP; (d) Fmoc-Ile-OH/HATU/TMP; (e) $(AcO)_2O$/DMAP; (f) AcOH/2,2,2-trifluoroethanol/DCM; (g) AMC/HATU/TMP; (h) 95% TFA/water).

sin.[14,15] The coupling reagent HATU with TMP as a catalytic base was very useful in building up the protected tetrapeptide, providing a product in high yield and high enantiomeric purity. It was intended to utilize **6** for coupling reactions via a free carboxyl group at aspartic acid with a series of fluorescent aromatic amines (coumarin- and quinolin-derivatives). The HATU/TMP procedure was also chosen for this reaction as it was previously reported that HATU is very efficient in solution synthesis,[5,16–18] while the highly hindered base TMP was particularly useful in the segment couplings as well.[5] The reaction was first attempted with the commercially available aromatic amine AMC. A 2 equiv excess of HATU and TMP, and 1.33 equiv excess of tetrapeptide relative to amine were used as a standard protocol. The protected AMC-conjugate was obtained with good yields (~75%). Deprotection was straightforward in trifluoroacetic acid (TFA)/water. Combustion analysis of the final products **7** and **8** indicated that the product was pure. However, the RP-HPLC chromatogram showed that the product consisted of two major peaks in a ratio of 1:3 (Fig. 1).

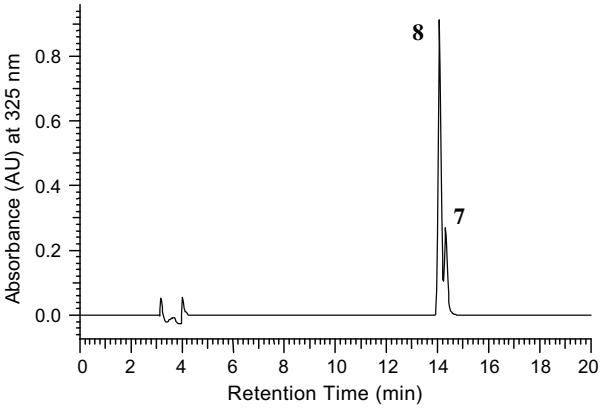


Figure 1. RP-HPLC-Chromatogram of the partially purified product containing **7** and **8** (Ac-Ile-Glu-Thr-D-Asp-AMC **8**: $t_R = 14.08$ min; Ac-Ile-Glu-Thr-L-Asp-AMC (**7**): $t_R = 14.32$ min. HPLC conditions: System 4, see Section 4).

Comparison of the HPLC retention time of commercially available Ac-IETD-AMC and our crude product revealed that the smaller peak (compound **7**) at t_R of 14.32 min was in agreement with the standard. The crude product was purified with analytical RP-HPLC and both compounds were isolated. Identical MS results of **7** and **8** clearly indicated that they were isomers. Decisive information about their absolute configuration came from CD studies with the two compounds (Fig. 2). As the strong absorption at wavelength of 325 nm was due to the presence of a coumarin chromophore, the differences in both spectra were most probably caused by the amino acid closest to the AMC moiety, that is, the aspartic acid. This assumption was further confirmed after incubation of both compounds with recombinant human caspase-8. In contrast to **7**, compound **8** was not hydrolyzed by the enzyme (data not shown). It is well known that caspases have strict requirements for L-aspartic acid to show any activity.[19]

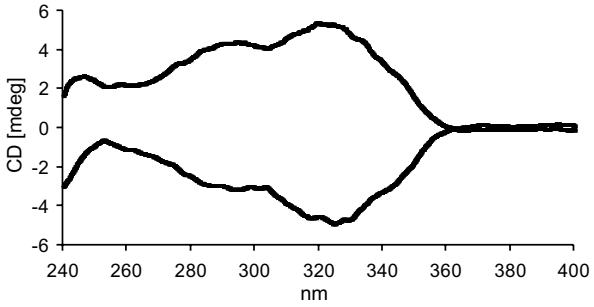


Figure 2. CD Spectrum of **7** (lower trace) and **8** (upper trace).

It was thus evident that the reaction of **6** and AMC in the presence of a 2 equiv excess of HATU and TMP resulted in facile epimerization at the aspartic acid. Owing to the fact that 75% of the D-Asp containing diastereoisomer was found in the product, asymmetric induction[2,3] during the coupling process appears likely since a large excess of the opposite epimer is seldom found in the literature.[†] The same epimerization reaction, while not to such a degree, was observed when Ac-Asp(*t*-Bu)-Met-Gln-Asp(*t*-Bu)-OH **9** (specific Caspase-3 sequence) was coupled with AMC to give 55% of the D-isomer.

2.2. Optimization of the AMC coupling reaction to reduce epimerization

To avoid epimerization, other coupling methods were investigated. The use of *N*,*N*′-dicyclohexylcarbodiimide (DCCI)[22] in the presence of 1-hydroxy-benzotriazole (HOBt) was found to be impractical as dicyclohexylurea (DCU) precipitated in DMF soon after all the reaction components had been added. Even with subsequent filtration of DCU, the desired product could not be detected. Replacement of DCCI with more soluble diisopropylcarbodiimide (DIPCI)[23] resulted in a product but with very poor yields and enantiomeric purity. The mixed-anhydride-producing coupling reagent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ)[24] not only caused a high degree of epimerization at the aspartic acid but also many unidentified side products were found. In addition, the racemization suppressive properties reported for $CuCl_2$[25] as an additive to the HATU procedure could not be confirmed in our study. Comparable results to the HATU/TMP procedure were only achieved with another mixed-anhydride coupling reagent, isobutyl chloroformate.[26]

Since the diastereoisomers of protected Ac-DMQD-AMC **10** and **11** were better separated with RP-HPLC than those of Ac-IETD-AMC, we decided to investigate these particular reaction conditions in more detail. A variety of factors that could influence the enantiomeric purity of the products formed were investigated: the coupling reagent, the

[†] Carpino had found 61.5% of D-isomer after coupling of Bz-Val-OH with H-Val-OMe using DCCI.[20] Benoiton et al. have found 74.7% of D-isomer after coupling of Z-Gly-Arg(Mtr)-OH with H-Val-OBzl·HCl using DCCI/HOBt.[21]

base, the pre-activation time and the solvent. After 48 h, each reaction was analyzed with RP-HPLC to estimate the degree of epimerization (Fig. 3). The coupling yield was calculated with a calibration curve for pure, *t*-Bu-protected Ac-DMQD-AMC **10**.

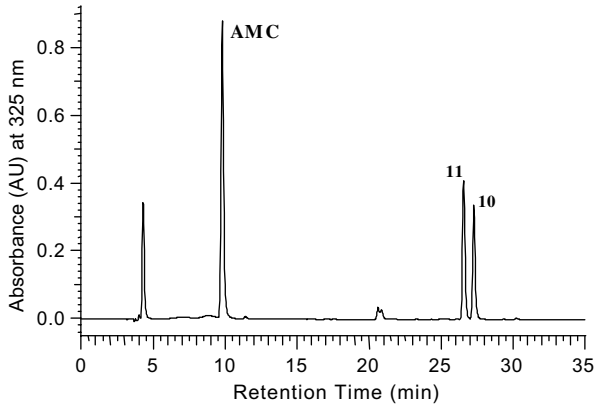


Figure 3. Representative HPLC chromatogram obtained with the model RP-HPLC system used to study aspartate epimerization during coupling of *t*-Bu protected Ac-DMQD-OH **9** with**AMC** (Ac-Asp(*t*-Bu)-Met-Gln-D-Asp(*t*-Bu)-AMC **11**: $t_R = 26.45$ min; Ac-Asp(*t*-Bu)-Met-Gln-L-Asp(*t*-Bu)-AMC **10**: $t_R = 27.14$ min. HPLC conditions: System 2, see Section 4).

To activate the carboxy group and accelerate the coupling reaction, a pre-activation step is often described in standard protocols; however, this approach has its disadvantages because the exposure of the activated species to the base can result in epimerization due to direct enolization or oxazolone formation.[27] Thus, we have shortened the pre-activation time from 10 min to 1 min and compared the degree of epimerization (Table 1, Nos. 1 and 2). In this case, the pre-activation time seemed to have no influence on epimerization, probably due to the relatively slow coupling rate (i.e., the reaction needs at least 48 h to be completed) and all further studies were done without pre-activation. It was also interesting to see how the rate of addition of the base influences the epimerization; however, such studies have not yet been performed.

We also investigated a series of hindered bases for their utility in this reaction (Table 1, Nos. 2–7). The most popular amine bases in peptide synthesis; that is, diisopropylcarbodiimide (DIEA, pK_a 10.98[‡]), 4-dimethylamino-pyridine (DMAP, pK_a 9.52[‡]) or*N*-methylmorpholine (NMM, pK_a 7.41[‡]), did not show any clear advantages over TMP (pK_a 7.33[‡]) in terms of reducing the extent of epimerization. On the other hand, the very hindered bases 2,6-dichloropyridine (DCP, pK_a −3.98[‡]) and TBP (pK_a 6.92[‡]) dramatically decreased the level of epimerization, probably due to both their reduced basicity and the steric hindrance of the basic nitrogen. The use of these bases, however, also led to a reduction in the overall yields. Nevertheless, TBP seemed to be a promising agent, and further studies with this compound were carried out to optimize its use.

[‡] Calculated by using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994–2007 ACD/Labs) as implemented in SciFinder Scholar 2007.

Table 1. Influence of preactivation time and base on aspartate epimerization estimated by HPLC[a]

No.	Base	Chemical yield (%)	L-Epimer yield (%)	D/(L + D) (%)
1	TMP/preact	79.1	27.8	54.9
2	TMP	78.7	27.9	54.3
3	DIEA	85.3	30.2	55.3
4	NMM	76.8	29.7	55.4
5	DMAP	61.1	25.8	50.9
6	TBP	13.5	12.9	4.9
7	DCP	11.9	11.1	6.8

[a] Mean of 1 to 3 independent determinations. Conditions: to a solution of **9** (1), HATU (2) and appropriate base (2) in DMF, AMC (1) was added after 10 min (No. 1) or 1 min (Nos. 2–7). HPLC chromatograms were recorded after 48 h (System 2, see Section 4).

Racemization is almost exclusively a base-induced side reaction, thus we postulated that the amount of base could play a major role in the outcome of this reaction. Indeed, a simple reduction of TMP used from 2 equiv to 0.5 (relative to carboxy component) getting reduced the level of epimerization (Table 2, Nos. 8–11). This trend was common for all the bases studied at in this work.

Due to the generally poor solubility of the protected peptides, DMF was initially used as a solvent. Dilution of DMF with a non-polar solvent, such as dichloromethane (DCM), also led to a lowering in the degree of epimerization without affecting the yields when TMP was used (Table 2, Nos. 8–16). This effect was in accordance with previously reported findings.[28,29] Despite acceptable levels of D-epimer achieved with 0.5 mol TMP in DMF/DCM, the overall yield of 36% was somewhat disappointing.

2.3. Optimization of AMC coupling reaction to improve synthetic yields

To improve the yield without compromising the enantiomeric purity, we employed the even more hindered base TBP. This base decreased the degree of aspartate epimerization even when used in excess, but the yields were lower when the reaction was carried out in DMF as already mentioned (Table 2, Nos. 17–19). This may be the reason why this base has never found an application in peptide chemistry, despite an intensive search for new, sterically hindered bases in this area.[30] It is notable that the use of TBP in the mixture of DMF/DCM not only reduced the level of epimerization, but also increased the yield by about 3-fold compared to the results obtained with DMF as the sole solvent (Table 2, Nos. 17–22). In fact, the system consisting of a 2 M excess of TBP in a 1:1 mixture of DMF/DCM (Table 2, No. 21) was the best compromise in terms of both yield and enantiomeric purity. TBP has previously been reported to be useful in glycosylation reactions and in the formation of vinyl triflates.[31] This study shows that TBP can be successfully applied to couple the peptides with weakly nucleophilic amines. TBP also has a practical advantage over TMP as it is a stable solid.

Table 2. Influence of the amount of base or solvent on aspartate epimerization estimated by HPLC[a]

No.	Base	Solvent	Chemical yield (%)	L-Epimer yield (%)	D/(L + D) (%)
8	TMP (4)[b]	DMF	61.3	26.0	54.6
9	TMP (2)	DMF	78.6	34.6	53.6
10	TMP (1)	DMF	59.0	38.0	28.8
11	TMP (0.5)	DMF	26.2	22.9	10.1
12	TMP (4)	DMF/DCM (1:1)	81.4	36.7	53.4
13	TMP (2)	DMF/DCM (1:1)	80.8	47.4	39.7
14	TMP (1)	DMF/DCM (1:1)	59.0	48.5	12.4
15	TMP (0.5)	DMF/DCM (1:1)	35.6	33.2	5.5
16	TMP (0.25)	DMF/DCM (1:1)	20.2	19.8	1.9
17	TBP (4)	DMF	17.9	16.7	6.5
18	TBP (2)	DMF	13.5	12.9	4.9
19	TBP (1)	DMF	8.6	8.2	4.2
20	TBP (4)	DMF/DCM (1:1)	60.8	50.5	6.4
21	**TBP (2)**	**DMF/DCM (1:1)**	**56.1**	**50.4**	**3.9**
22	TBP (1)	DMF/DCM (1:1)	35.4	33.8	2.2

[a] Mean of 1 to 3 independent determinations.
[b] The numbers in parentheses refer to the number of equivalents of base. Conditions: to a solution of **9** (1), HATU (2) and base in appropriate solvent, AMC (1.5) was added after 2 min. HPLC chromatograms were recorded after 48 h (System 2, see Section 4).

We have also studied the utility of other aminium-based coupling reagents such as: *N*-[(1*H*-benzotriazol-1-yl) (dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU[32] and the relatively new *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium-5-chloro-, hexafluorophosphate *N*-oxide HCTU[33] and compared these to HATU in this particular reaction (Table 3, Nos. 23–29). The results showed that HATU was superior to HBTU in terms of yield and purity, which was in accordance with previous reports.[4] HCTU and HATU gave similar levels of epimerization, but HATU was slightly better in terms of yield.

The amount of HATU used and its influence on the reaction was also investigated (Table 3, Nos. 30–35). It was found that an excess of this coupling reagent would accelerate the reaction without significantly sacrificing the enantiomeric purity. However, in cases where the enantiomeric purity of the product was very poor, HATU could in fact decrease the level of epimerization when it was used in excess. Thus, a 2 M excess of HATU was routinely used.

In most cases, when linear peptides are built by incorporation of single amino acid residues, especially in solid-phase

Table 3. Effects of HATU, HCTU and HBTU on aspartate epimerization estimated with HPLC[a]

No.[b]	AMC	Coupling reagent	Base	Solvent	Chemical yield (%)	L-Epimer yield (%)	D/(L + D) (%)
23	1.5	HATU (2)[c]	TMP (0.5)	DMF	26.2	22.9	10.1
24	1.5	HCTU (2)	TMP (0.5)	DMF	23.7	20.8	8.7
25	1.5	HBTU (2)	TMP (0.5)	DMF	18.2	14.5	20.0
26	1.5	HATU (2)	TMP (0.5)	DMF/DCM (1:1)	35.6	33.2	5.5
27	1.5	HCTU (2)	TMP (0.5)	DMF/DCM (1:1)	26.4	27.1	3.2
28	1.5	HATU (2)	TBP (2)	DMF/DCM (1:1)	56.1	50.4	3.9
29	1.5	HCTU (2)	TBP (2)	DMF/DCM (1:1)	43.1	40.4	4.1
30	1	HATU (1)	TMP (1)	DMF	32.8	16.6	43.6
31	1	HATU (2)	TMP (1)	DMF	42.5	23.3	34.6
32	2	HATU (1)	TMP (0.5)	DMF	18.3	17.5	4.6
33	2	HATU (2)	TMP (0.5)	DMF	42.3	39.0	6.0
34	2	HATU (1)	TBP (2)	DMF/DCM (1:1)	34.0	30.5	4.5
35	2	HATU (2)	TBP (2)	DMF/DCM (1:1)	40.5	34.1	5.1

[a] Mean of 1 to 3 independent determinations.
[b] Some reactions are repeated with new number from other tables to allow direct comparisons.
[c] The numbers in parentheses refer to the number of equivalents. Conditions: to a solution of **9** (1), coupling reagent and base in appropriate solvent, AMC (1.5) was added after 2 min. HPLC chromatograms were recorded after 48 h (System 2, see Section 4).

Table 4. Influence of the amount of AMC on aspartate epimerization estimated with HPLC[a]

No.[b]	AMC	HATU	Base	Solvent	Chemical yield (%)	L-Epimer yield (%)	D/(L + D) (%)
36	1[c]	2	TMP (1)	DMF	42.5	23.3	34.6
37	1.5	2	TMP (1)	DMF	59.0	38.0	28.8
38	2	2	TMP (1)	DMF	56.9	45.1	15.7
39	1	2	TMP (1)	DMF/DCM (1:1)	51.8	36.2	21.4
40	1.5	2	TMP (1)	DMF/DCM (1:1)	59.0	48.5	12.4
41	2	2	TMP (1)	DMF/DCM (1:1)	60.7	49.9	13.3
42	1	1	TMP (1)	DMF	32.8	16.6	43.6
43	1.5	1	TMP (1)	DMF	50.2	31.8	32.7
44	2	1	TMP (1)	DMF	41.6	30.7	20.7
45	1	1	TMP (1)	DMF/DCM (1:1)	51.1	33.0	29.7
46	2	1	TMP (1)	DMF/DCM (1:1)	50.8	40.5	19.6
47	1	2	TBP (2)	DMF/DCM (1:1)	40.5	34.1	5.1
48	1.5	2	TBP (2)	DMF/DCM (1:1)	56.1	50.4	3.9
49	2	2	TBP (2)	DMF/DCM (1:1)	44.3	41.1	2.7

[a] Mean of 1 to 3 independent determinations.
[b] Some reactions are repeated with new number from other tables to allow direct comparisons.
[c] The numbers refer to the number of equivalents used. Conditions: to a solution of **9** (1), HATU and base in appropriate solvent, AMC was added after 2 min. HPLC chromatograms were recorded after 48 h (System 2, see Section 4).

synthesis, an excess of acylating agent is recommended. In this case, the more precious amino component should be completely utilized. This approach is still followed even when the carboxy component is more valuable.[34] In our case, this strategy was not desirable because the excess of the poorly soluble protected tetrapeptide (especially **6**) requires further dilution of the reaction mixture, which could decrease the coupling rate of the reaction. Instead, we used an excess of less valuable amino component AMC, as we found that the unreacted starting material could be easily removed by washing the product with diethyl ether. The use of an excess of aromatic amine brought an additional benefit as the yield could be increased in most cases (Table 4). Moreover, the level of epimerization could be significantly reduced. The use of a 1.5-fold excess of amine was found to be more practical than a 2-fold excess, because not all aromatic amines used could be efficiently extracted with diethyl ether (data not shown). It should be noted that the application of an excess of amine component (as well as the aminium coupling reagent) can be associated with the formation of undesired guanidinium products.[7] However, we did not observe this type of compounds during our study.

2.4. Mechanistic features of the coupling and epimerization reactions

Additional studies were carried out to understand the mechanistic features of the epimerization reaction. We found that tetrapeptide **6** as well as **9** undergo epimerization during the coupling almost to the same degree as the products formed (dependent on basicity or polarity of the solution). The tetrapeptides are stable for several days in DMF as well as in the mixture with HATU in DMF, or TMP in DMF. This indicated that as long as the tetrapeptide was not activated, direct proton abstraction and loss of chiral integrity of the substrate did not occur. The epimerization of the tetrapeptides could be associated with the presence of water, which would compete with weakly nucleophilic AMC and hydrolyzed the activated racemized species (probably active ester) back to the enatiomerically inactive substrate. However, this hypothesis could not be confirmed as we found that the reaction of **6** with AMC by means of HATU/TMP in totally anhydrous DMF under nitrogen did not change the level of epimerization of either the product or the substrate.

Additional experiments also revealed that diastereomers **7** and **8** are interconvertable at elevated temperatures in an inert solvent. For example, with RP-HPLC it was found that pure **8** converts specifically to **7** when heated at 110 °C in DMSO (Fig. 4). After about 8 h of heating, an equilibrium was established, whereby **8** remains in the excess over **7** (ratio ~55:45). The compounds were not isolated from the solution but their t_R fit perfectly to those found for pure **7** and **8**. Moreover, the heating of **7** causes its conversion to **8** and after 22 h of heating; in fact, the RP-HPLC profile looks virtually identical as in the previous case (not shown). Interestingly, the D-Asp containing epimer **8** seems to be thermodynamically more stable than

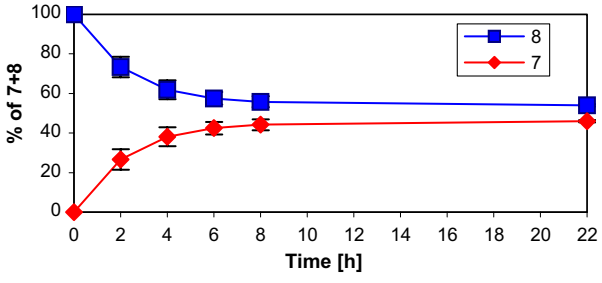


Figure 4. Kinetics of conversion of **8** into **7** in DMSO at 110 °C.

the L-epimer **7** since it is present in excess at equilibrium, regardless of which compound is initially heated.

The most probable mechanism by which aspartate epimerization could occur is via a succinimide (also called aspartimide) intermediate formation.[35,36] The succinimides are very vulnerable to base-catalyzed epimerization, presumably through the α-proton abstraction–enolization mechanism.[27] Asparic acid (and asparagine) forms aspartimides by nucleophilic attack of the β-carbonyl group by the NH-group of the downstream (C-terminal) peptide bond resulting in the formation of D-aspartate isomers and isoaspartyl residues. The rate of the formation of the succinimides is dependent upon factors that increase the deprotonation of the peptide nitrogen.[37] The aromatic system of AMC having electron drawing properties might contribute to the discussed effect. The flexibility of the short tetrapeptide usually allows the dihedral bond angles necessary for succinimide to form.[36] These very interesting phenomena have also been observed after heating commercially available compounds such as Ac-DMQD-AMC (obtained after deprotection of **10**) or Ac-IETD-*p*NA (*p*NA = *p*-nitroanilide) (not shown).

The D-diastereoisomer was not the only by-product that we observed during this study. Under the conditions associated with strong aspartate epimerization, considerable amounts of another side product were detected by HPLC. We were able to isolate this new side-product after the reaction of **6** with HATU and TMP (without AMC). The NMR and LC–MS analysis revealed that the free carboxy group of tetrapeptide has been converted to the corresponding *N*,*N*-dimethylamide **12** (COOH → CON$(CH_3)_2$). This unexpected result prompted us to study this side reaction in more detail. RP-HPLC analysis showed that the formation of the amide is detectable within minutes (Fig. 5) and that the new product undergoes epimerization as well.

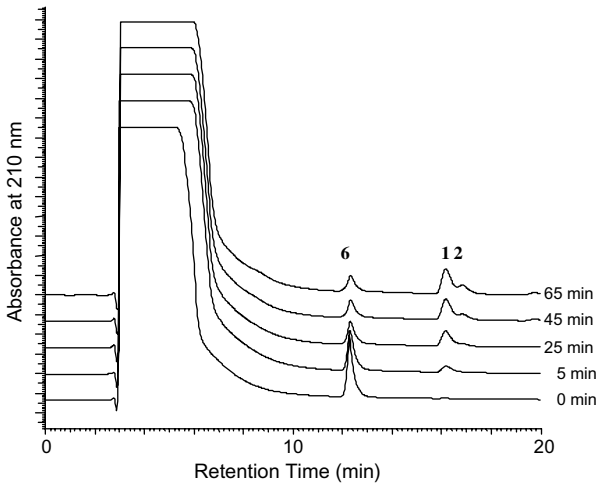


Figure 5. Conversion of **6** to the corresponding *N*,*N*-dimethylamide **12** after the reaction with HATU/TMP (To a solution of **6** (1) and HATU (1) in DMF ($t = 0$ min) the equimolar amount of TMP was added and the reaction mixture was analyzed at appropriate time. **6**: $t_R = 12.32$ min, **12**: $t_R = 16.16$ and 16.83 min. HPLC conditions: System 5, see Section 4).

There are at least three possibilities that could lead to the formation of the *N*,*N*-dimethyl derivative **12**: (1) It might have been generated from trace amounts of dimethylamine in commercial HATU.[38] (2) Contamination of DMF with trace amounts of dimethylamine could have been responsible for the formation of **12**. (3) Eventually the decomposition of aminium salt under coupling conditions could also be the cause for the formation of **12**.[39] However, we could not clarify which mechanism governed this side reaction. Nevertheless, the dilution of reaction mixture with DCM (optimized reaction conditions) was sufficient enough to suppress the formation of **12** and the problem was solved.

3. Conclusion

The results of our study show that epimerization during segment coupling with weakly nucleophilic aromatic amines should not be underestimated. Furthermore, when coupling reagents such as HATU in DMF are used, *N*,*N*-dimethylamide side-products also form, leading to noticeable reductions in yields. These side reactions can, however, be minimized by carefully optimizing the reaction conditions. It is reported here for the first time, that the hindered base TBP was superior to TMP during the coupling with HATU. Moreover, an excess of the less precious amine improves the enantiomeric purity and yield of the products. This newly developed synthetic method has now been successfully employed to prepare a variety of novel caspase-8 and caspase-3 substrates.[40]

4. Experimental

4.1. General

All reagents and solvents were obtained from commercial sources. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker Avance 600 or on a DPX200 spectrometer in DMSO-d_6. Mass spectra were taken on Finnigan MAT 95 Spectrometer (ESI) (**7**, **8**) or microTOF 112 (ESI) (remaining compounds). CD spectra were recorded on JASCO-J 810 Circular Dichrosm Chiroptical Spectrometer (the compounds have been analyzed at the concentration of 0.1 g/L in water). RP-HPLC was accomplished by using a Merck-Hitachi system consisting of a D-7000 interface, L-7100 pump, L-7360 thermostat column (30 °C), L-7612 solvent degasser, L-4500 diode-array-detector and a 7125 Rheodyne sample injector fitted with a 20 μL injection loop. All separations were carried out with a CC 250/4 Nucleosil 120-5 C_{18} column (Macherey–Nagel). Flow rate: 0.7 mL/min. Mobile phases: A: Acetonitrile + 0.1% TFA; B: Water + 0.1% TFA; C: Methanol + 0.1% TFA. System 1: 30–65% A/B in 60 min; System 2: 25–60% A/B in 35 min; System 3: 40–80% C/B in 45 min; System 4: 20–47% A/B in 20 min; System 5: 50% A/B.

4.2. Analytical procedure used to examine aspartate epimerization with RP-HPLC

At room temperature, 0.003 mmol of Ac-Asp(*t*-Bu)-Met-Gln-Asp(*t*-Bu)-OH **9** and the appropriate amount of

coupling reagent, base and AMC were reacted for 48 h (final volume of 70–90 μL) under the conditions indicated at footnotes of Tables 1–4. Each reaction was directly analyzed with RP-HPLC (System 2) (2 μL of the reaction mixture was diluted with 100 μL acetonitrile and 398 μL water before injection). The chemical yields were calculated from the calibration curve (area under the peak) for the pure product Ac-Asp(*t*-Bu)-Met-Gln-L-Asp(*t*-Bu)-AMC **10**. The by-products (if any) with $\lambda_{max} \sim 325$ nm (AMC conjugate) were also included for the calculation of the chemical yields. The level of epimerization was calculated with the equation shown in the Tables, where L and D represent the peak area of L- and D-diastereoisomers, respectively. The accuracy of the method was confirmed by the true synthesis of **10** where 49% yield and 4.2% of the D-isomer was found (compare with Table 2, No. 21).

4.3. General protocol for the solid-phase synthesis of *t*-Bu-protected tetrapeptides Ac-X-X-X-D-OH

All reactions were done on a manually operated nitrogen-stirred solid-phase peptide synthesis reactor consisting of a sintered glass column and three-way tap at room temperature. Fmoc-amino acid (0.75 mmol) and HATU or HCTU (0.75 mmol) were dissolved in DMF (4 mL) and cooled to 0 °C. TMP (0.75 mmol) was added and the resulting solution was stirred for 10 min at 0 °C and for a further 10 min at 20 °C. The solution was transferred to the solid-phase reactor, which contained about 0.5 mmol resin bound aspartic acid ester (H-Asp(*t*-Bu)-2ClTrt resin, 75–150 μm, ~1 mmol/g, Fluka). The resin was previously swollen with DMF. The reaction was allowed to proceed for 90 min, then the reaction solution was drawn off and the resin washed twice with DMF (5 mL/0.5 g resin). The procedure was repeated with the same amount of reagents until the reaction was completed (about 60–90 min). The completion of the coupling reactions was verified by the Kaiser ninhydrin test.[41]

4.4. Cleavage of the Fmoc-group

The cleavage was performed with 20% piperidine in DMF for 5 min at room temperature followed with the second treatment for another 5 min.

4.5. Acetylation of the final amino acid

After the cleavage of the Fmoc-group from the final amino acid and following standard washing, 4 mL of DMF was added to the resin. Then 6 equiv of $(AcO)_2O$ were added followed by a solution of DMAP in DMF (0.1 equiv, 50 mM). After 45 min, the reaction was drawn off and washed.

4.6. Washing procedure

After each coupling step, removal of the Fmoc-group or acetylation, the resin was washed with 5 mL/0.5 g resin of the following solvents: DMF (3×), isopropanol (2×) and DMF (3×). At the end of the synthesis and before cleavage of the tetrapeptide from the resin, additional washings with DCM (2 × 5 mL) were performed.

4.7. Cleavage of the protected tetrapeptides from the 2-chlorotrityl resin

The peptide resin ester (0.5 g) was stirred with 10 mL of the mixture AcOH/TFE/DCM (2:2:6) for 30 min and the resin was then filtered and washed once with 5 mL DCM. This procedure was repeated four times and the combined filtrates were concentrated in vacuo. The resulting solution was diluted 1:1 with toluene and once again concentrated in vacuo to remove acetic acid. The protected tetrapeptide was precipitated with ice cold ether, filtered and washed with diethyl ether. The product can be reprecipitated with TFE/water to remove any remaining acetic acid, washed with diethyl ether, and finally dried in vacuo.

4.7.1. Ac-Ile-Glu(*t*-Bu)-Thr(*t*-Bu)-Asp(*t*-Bu)-OH 6. Yield 300 mg (87.5%); ^{1}H NMR (200 MHz, DMSO-d_6) $\delta = 12.81$ (br s, Asp-COOH), 8.13 (d, $J = 7.8$ Hz, Glu-NH), 8.00 (d, $J = 7.8$ Hz, Asp-NH), 7.90 (d, $J = 8.6$ Hz, Ile-NH), 7.60 (d, $J = 8.2$ Hz, Thr-NH), 4.55 (m, Asp-C_α-H), 4.34 (m, Glu-C_α-H), 4.27 (m, Thr-C_α-H), 4.21 (m; Ile-C_α-H), 3.89 (m, Thr-C_β-H), 2.65 (m, Asp-C_β-H_2), 2.20 (m, Glu-Cγ-H_2), 1.90 (m, Glu-C_β-H1), 1.85 (s, Ac-Ile), 1.75 (m, Glu-C_β-H2), 1.70 (m, Ile-C_β-H), 1.41 (m, Ile-C_γ-H1), 1.40 (s, Asp-*t*-Bu, Glu-*t*-Bu), 1.12 (s, Thr-*t*-Bu), 1.1 (m, Ile-C_γ-H2), 1.0 (d, $J = 6.2$ Hz, Thr-CH_3), 0.82 (d, Ile-C_β-CH_3), 0.79 (t, Ile-C_γ-CH_3); ^{13}C NMR (50 MHz, DMSO-d_6) $\delta = 171.9$, 171.5, 170.9, 169.5, 169.2, 169.0, 80.5 (C-*t*-Bu), 79.8 (C-*t*-Bu), 74.1 (C-*t*-Bu), 67.0 (C_βH-Thr), 57.2 (C_αH-Thr), 56.8 (C_αH-Ile), 51.9 (CH-Glu), 48.7 (CH-Asp), 37.4 (CH_2-Asp), 36.6 (C_βH-Ile), 31.4 ($C_\gamma H_2$-Glu), 28.1 (3 × CH_3-*t*-Bu), 27.9 (3 × CH_3-*t*-Bu), 27.8 (3 × CH_3-*t*-Bu), 27.1 ($C_\beta H_2$-Glu), 24.5 (CH_2-Ile), 22.6 (CH_3-acetyl), 18.4 (CH_3-Thr), 15.5 (C_β-CH_3-Ile), 11.1 (C_γ-CH_3-Ile); HPLC, System 1: $t_R = 37.63$ min; System 3: $t_R = 41.28$ min; no detectable amounts of the D-isomer, purity: 99.8% at $\lambda = 215$ nm; MS (ESI) *m*/*z* calcd for $C_{33}H_{58}N_4O_{11}$: 686.8. Found: *m*/*z* 687.4 $(M+H)^+$, 631.4 $(M-(t\text{-Bu})+H)^+$, 575.3 $(M-(2 \times t\text{-Bu})+H)^+$, 519.2 $(M-(3 \times t\text{-Bu})+H)^+$; The structure was further identified by DEPT and 2D NMR (COSY, HSQC, HMBC) spectra.

4.7.2. Ac-Asp(*t*-Bu)-Met-Gln-Asp(*t*-Bu)-OH 9. Yield: 311 mg (94%); ^{1}H NMR (200 MHz, DMSO-d_6) $\delta = 12.76$ (br s, Asp2-COOH), 8.18 (m, Asp1-NH, Asp2-NH), 7.99 (d, $J = 7.8$ Hz, Gln-NH), 7.90 (d, $J = 7.8$ Hz, Met-NH), 7.19 (s, Gln-NH_2), 6.73 (s, Gln-NH_2), 4.59-4.52 (m, Asp1-C_α-H, Asp2-C_α-H), 4.30 (m, Met-C_α-H), 4.22 (m, Gln-C_α-H), 2.61 (m, Asp2-C_β-H_2), 2.40 (m, Asp1-C_β-H_2), 2.40 (m, Met-C_γ-H_2), 2.11 (m, Gln-C_γ-H_2), 2.01 (s, Met-CH_3), 1.88 (m, Met-C_β- H_2), 1.86 (m, Gln-C_β-H_2), 1.83 (s, Gln-CH_3), 1.37 (m, Asp1-*t*-Bu, Asp2-*t*-Bu); ^{13}C NMR (50 MHz, DMSO-d_6) $\delta = 173.6$, 171.9, 170.8, 170.5, 170.4, 169.32, 169.29, 169.0, 80.3 (C-*t*-Bu), 80.1 (C-*t*-Bu), 51.9 (CH-Met), 51.8 (CH-Gln), 49.5 (CH-Asp), 48.5 (CH-Asp), 37.2 (CH_2-Asp), 37.1 (CH_2-Asp), 31.8 (CH_2-Gln), 31.3 (CH_2-Gln), 29.3 (CH_2-Met), 27.9 (CH_2-Met), 27.61 (3 × CH_3-*t*-Bu), 27.58 (3 × CH_3-*t*-Bu), 22.4 (CH_3-acetyl), 14.5 (CH_3-Met); HPLC, System 2: $t_R = 16.40$ min; System 3: $t_R = 23.41$ min; no detectable amounts of the D-isomer, purity: 98.26% at $\lambda = 215$ nm. The structure

was further identified by DEPT and 2D NMR (COSY, HSQC) spectra.

4.8. Optimized coupling procedure of *t*-Bu-protected tetrapeptides with AMC

After dissolving 0.1 mmol of tetrapeptide, 0.2 mmol of HATU and 0.2 mmol of TBP in 1 mL DMF, the resulting solution was stirred for 5 min at 20 °C. AMC was added (0.15 mmol/200 μL DMF) and the reaction mixture was immediately diluted with 600 μL DCM. After 5 days, the solution was transferred to 50 mL ethyl acetate and washed three times with 1 M HCl, 1 M $NaHCO_3$ and saturated NaCl in water. After drying with sodium sulfate, the organic layer was evaporated in vacuo to afford a white-yellow solid. The precipitate was washed with diethyl ether to remove any remaining AMC and dried. As indicated by NMR and RP-HPLC studies, the product was sufficiently pure and could be directly deprotected without further purifying.

4.9. Deprotection procedure

Ac-Ile-Glu(*t*-Bu)-Thr(*t*-Bu)-Asp(*t*-Bu)-AMC was dissolved in ice cold 3 mL 95% TFA/water and reacted for 60 min at room temperature. Due to the presence of methionine in Ac-Asp(*t*-Bu)-Met-Gln-Asp(*t*-Bu)-AMC **10** an ice cold cleavage mixture contained 2.25 mL TFA, 0.45 mL EDT, 0.15 g *p*-cresol and 0.15 mL water was used. The resulting mixture was poured into 30 mL of ice cold diethyl ether with vigorous stirring. The precipitate was collected by filtration and washed with diethyl ether to provide a white solid. The product was purified by analytical RP-HPLC and lyophilized.

4.9.1. Ac-Ile-Glu-Thr-Asp-L-AMC 7. From **6** and **AMC**. Yield 16.5 mg (46%); NMR spectrum was recorded only for the commercially available material. All other analytical data come from synthesized **7**. ^{1}H NMR (600 MHz, DMSO-d_6) $\delta = 10.08$ (s, AMC-NH), 8.36 (d, Asp-NH), 8.19 (d, Glu-NH), 7.93 (d, Ile-NH), 7.92 (d, Thr-NH), 7.77 (s, 9-AMC), 7.70 (d, 6-AMC), 7.58 (d, 7-AMC), 6.27 (s, 3-AMC), 5.11 (br s, Thr-OH), 4.63 (m, Asp-C_α-H), 4.36 (m, Glu-C_α-H), 4.17 (m; Ile-C_α-H), 4.15 (m, Thr-C_α-H), 4.05 (m, Thr-C_β-H), 2.82 (impurity from commercial comp.), 2.63 (m, Asp-C_β-H_2), 2.40 (s, AMC-CH_3), 2.28 (m, Glu-Cγ-H1), 2.22 (m, Glu- Cγ-H2), 1.92 (m, Glu-C_β-H1), 1.85 (s, Ac-Ile), 1.81 (m, Glu-C_β-H2), 1.68 (m, Ile-C_β-H), 1.41 (m, Ile-C_γ-H1), 1.07 (m, Ile-C_γ-H2), 1.07 (m, Thr-CH_3), 0.81 (d, Ile-C_β-CH_3), 0.78 (t, Ile-C_γ-CH_3); ^{13}C NMR (150 MHz, DMSO-d_6) $\delta = 174.1$, 171.9, 171.8, 171.2, 170.3, 169.7, 169.2, 159.9 (2-AMC), 153.6 (5-AMC), 152.9 (10-AMC), 142.0 (8-AMC), 125.8 (6-AMC), 115.3 (7-AMC), 115.1 (4-AMC), 112.3 (3-AMC), 105.7 (9-AMC), 66.2 (C_βH-Thr), 58.7 (C_αH-Thr), 56.7 (C_αH-Ile), 52.0 (CH-Glu), 50.8 (CH-Asp), 45.67 (impurity present in commercial comp.), 36.9 (CH_2-Asp), 36.4 (C_βH-Ile), 30.4 ($C_\gamma H_2$-Glu), 27.0 ($C_\beta H_2$-Glu), 24.4 (CH_2-Ile), 22.4 (CH_3-acetyl), 19.4 (CH_3-Thr), 17.9 (CH_3-AMC), 15.3 (CH_3-Ile), 10.9 (CH_3-Ile), 9.95 (impurity present in commercial comp.); HPLC, System 4: $t_R = 14.32$ min (identical with commercial comp.), 0.94% of the D-isomer in the crude product, <0.1% of the D-isomer in the purified product, purity: 99.7% at $\lambda = 325$ nm; MS (ESI) *m*/*z* calcd for $C_{31}H_{41}N_5O_{12}$: 675.7. Found: *m*/*z* 676.6 $(M+H)^+$. The structure was further identified by DEPT and 2D NMR (COSY, C-H COSY, NOESY) spectra.

4.9.2. Ac-Ile-Glu-Thr-D-Asp-AMC 8. From **6** and **AMC**. Compound **8** was isolated from a mixture of **7** and **8** before optimizing the reaction conditions. ^{1}H NMR (600 MHz, DMSO-d_6) $\delta = 12.25$ (br s; COOH), 10.00 (s, AMC-NH), 8.58 (d, Asp-NH), 8.19 (d, Glu-NH), 7.98 (s, 9-AMC), 7.95 (d, Ile-NH), 7.87 (d, Thr-NH), 7.73 (d, 6-AMC), 7.62 (d, 7-AMC), 6.29 (s, 3-AMC), 5.03 (br s, Thr-OH), 4.78 (m, Asp-C_α-H), 4.34 (dd, Glu-C_α-H), 4.17 (t; Ile-C_α-H), 4.05 (t, Thr-C_α-H), 3.88 (m, Thr-C_β-H), 2.85 (dd, Asp-C_β-H1), 2.63 (m, Asp-C_β-H2), 2.41 (s, AMC-CH_3), 2.26 (m, Glu-Cγ-H_2), 1.93 (m, Glu-C_β-H1), 1.86 (s, Ac-Ile), 1.77 (m, Glu-C_β-H2), 1.69 (m, Ile-C_β-H), 1.41 (m, Ile-C_γ-H1), 1.09 (m, Ile-C_γ-H2), 1.05 (d, Thr-CH_3), 0.81 (d, Ile-C_β-CH_3), 0.77 (t, Ile-C_γ-CH_3); ^{13}C NMR (150 MHz, DMSO-d_6) $\delta = 173.9$, 171.7, 171.6, 171.4, 170.1, 169.9, 169.4, 159.9 (2-AMC), 153.5 (5-AMC), 152.9 (10-AMC), 141.8 (8-AMC), 125.8 (6-AMC), 115.7 (4-AMC), 115.2 (7-AMC), 112.4 (3-AMC), 106.0 (9-AMC), 66.4 (C_βH-Thr), 59.6 (C_αH-Thr), 56.9 (C_αH-Ile), 51.8 (CH-Glu), 50.3 (CH-Asp), 36.2 (C_βH-Ile), 35.9 (CH_2-Asp), 30.0 ($C_\gamma H_2$-Glu), 26.8 ($C_\beta H_2$-Glu), 24.4 (CH_2-Ile), 22.4 (CH_3-acetyl), 19.5 (CH_3-Thr), 17.9 (CH_3-AMC), 15.3 (CH_3-Ile), 10.8 (CH_3-Ile); HPLC, System 4: $t_R = 14.08$ min; <0.1% of L-isomer, purity: 99.86% at $\lambda = 325$ nm; MS (ESI) *m*/*z* calcd for $C_{31}H_{41}N_5O_{12}$: 675.7. Found: *m*/*z* 676.6 $(M+H)^+$. The structure was also further identified by DEPT and 2D NMR (COSY, C–H COSY, NOESY) spectra.

4.9.3. Ac-Asp1(*t*-Bu)-Met-Gln-L-Asp2(*t*-Bu)-AMC 10. From **9** and **AMC** (without deprotection). Isolated from the reaction mixture of **10** and **11** before optimizing the reaction conditions. ^{1}H NMR (200 MHz, DMSO-d_6) $\delta = 10.31$ (s, NH-coum.), 8.33 (d, $J = 7.4$ Hz, Asp1-NH), 8.20 (d, $J = 7.8$ Hz, Asp2-NH), 8.12 (d, $J = 7.2$ Hz, Met-NH), 7.99 (d, $J = 7.8$ Hz, Gln-NH), 7.78 (d, $J = 1.8$ Hz, 9-coum.), 7.73 (d, $J = 8.8$ Hz, 6-coum.), 7.55 (dd, $J = 8.8$, 1.8 Hz, 7-coum.), 7.24 (s, Gln-NH_2), 6.77 (s, Gln-NH_2), 6.27 (d, $J = 0.8$ Hz, 3-coum.), 4.71 (m, Asp1-C_α-H), 4.58 (m, Asp2-C_α-H), 4.32 (m, Met-C_α-H), 4.19 (m, Gln-C_α-H), 2.75–2.30 (m, Asp2-C_β-H_2, Asp1-C_β-H_2), 2.41 (m, Met-C_γ-H_2), 2.36 (s, 11-coum.), 2.12 (m, Gln-C_γ-H_2), 2.00 (s, Met-CH_3), 1.91 (m, Met-C_β-H_2), 1.86 (m, Gln-C_β-H_2), 1.82 (s, Gln-CH_3), 1.36 (m, Asp1-*t*-Bu, Asp2-*t*-Bu); ^{13}C NMR (50 MHz, DMSO-d_6) $\delta = 173.6$, 171.12, 171.07, 170.6, 169.6, 169.35, 169.33, 168.9, 159.9 (2-coum.), 153.5 (5-coum.), 152.9 (10-coum.), 142.0 (8-coum.), 125.8 (6-coum.), 115.4 (7-coum.), 115.2 (4-coum.), 112.4 (3-coum.), 105.9 (9-coum.), 80.4 (C-*t*-Bu), 80.1 (C-*t*-Bu), 52.6 (CH-Gln), 52.0 (CH-Met), 50.6 (CH-Asp1), 49.5 (CH-Asp2), 37.2 (CH_2-Asp), 37.1 (CH_2-Asp), 31.6 (CH_2-Gln), 31.2 (CH_2-Gln), 29.3 (CH_2-Met), 27.60 ($3 \times CH_3$-*t*-Bu), 27.58 ($3 \times CH_3$-*t*-Bu), 27.4 (CH_2-Met), 22.4 (CH_3-acetyl), 17.9 (11-coum.), 14.5 (CH_3-Met); HPLC, System 1: $t_R = 28.96$ min; System 2: $t_R = 27.09$ min; Diastereomeric ratio: L/D: 99.71/0.29%; purity: 96.23% at

$\lambda = 325$ nm; MS (ESI) m/z calcd for $C_{38}H_{54}N_6S_1O_{12}$: 818.9. Found: m/z 817.3 $(M-H)^-$, 931.3 $(M+CF_3COO^-)^-$. The structure was further identified by DEPT and 2D NMR (COSY, HSQC) spectra.

4.9.4. Ac-Asp1(*t*-Bu)-Met-Gln-D-Asp2(*t*-Bu)-AMC 11. From **9** and **AMC** (without deprotection). Isolated from a mixture of **10** and **11** before optimizing the reaction conditions. ^{1}H NMR (200 MHz, DMSO-d_6) $\delta = 10.15$ (s, NH-coum.), 8.47 (d, $J = 8.0$ Hz, Asp2-NH), 8.22-8.18 (m, Asp1-NH, Met-NH), 8.00 (d, $J = 7.6$ Hz, Gln-NH), 7.87 (d, $J = 1.6$ Hz, 9-coum.), 7.75 (d, $J = 8.6$ Hz, 6-coum.), 7.59 (d, $J = 8.6$, 1.6 Hz, 7-coum.), 7.23 (s, Gln-NH_2), 6.76 (s, Gln-NH_2), 6.28 (d, $J = 0.8$ Hz, 3-coum.), 4.77 (m, Asp2-C_α-H), 4.59 (m, Asp1-C_α-H), 4.31 (m, Met-C_α-H), 4.15 (m, Gln-C_α-H), 2.90-2.40 (m, Asp2-C_β-H_2), Asp1-C_β-H_2), 2.42 (m, Met-C_γ-H_2), 2.40 (s, 11-coum.), 2.13 (m, Gln-C_γ-H_2), 1.97 (s, Met-CH_3), 1.89 (m, Met-C_β-H_2), 1.86 (m, Gln-C_β-H_2), 1.85 (s, Gln-CH_3), 1.35 (m, Asp1-*t*-Bu), 1.33 (m, Asp2-*t*-Bu); ^{13}C NMR (50 MHz, DMSO-d_6) $\delta = 173.5$, 171.3, 171.2, 170.6, 169.7, 169.4, 169.0, 168.9, 159.9 (2-coum.), 153.5 (5-coum.), 152.9 (10-coum.), 141.8 (8-coum.), 125.8 (6-coum.), 115.5 (7-coum.), 115.2 (4-coum.), 112.4 (3-coum.), 105.9 (9-coum.), 80.4 (C-*t*-Bu), 80.1 (C-*t*-Bu), 52.9 (CH-Gln), 51.9; (CH-Met), 50.3 (CH-Asp1), 49.5 (CH-Asp2), 37.3 (CH_2-Asp), 37.1 (CH_2-Asp), 31.6 (CH_2-Gln), 31.2 (CH_2-Gln), 29.3 (CH_2-Met), 27.60 ($3 \times CH_3$-*t*-Bu), 27.58 ($3 \times CH_3$-*t*-Bu), 27.2 (CH_2-Met), 22.4 (CH_3-acetyl), 17.8 (11-coum.), 14.5 (CH_3-Met); HPLC, System 1: $t_R = 27.87$ min; System 2: $t_R = 26.40$ min; MS (ESI) m/z calcd for $C_{38}H_{54}N_6S_1O_{12}$: 818.9. Found: m/z 817.3 $(M-H)^-$, 931.3 $(M+CF_3COO^-)^-$. The structure was further identified by DEPT and 2D NMR (COSY, HSQC, HMBC) spectra.

4.10. Aspartate epimerization under elevated temperatures with RP-HPLC

An approximately 1 mM solution of appropriate derivative in DMSO (final volume ~400 μL) was gradually heated in a closed test tube to the temperature of 110°. Compounds **7** and **8** were analyzed at time periods indicated in Figure 4. Routinely 5 μL of the reaction mixture was diluted with 120 μL of water before injection. Separations were done with a CC 250/4 Nucleosil 120-5C_{18} column (System 4).

4.11. Synthesis of Ac-Ile-Glu(*t*-Bu)-Thr(*t*-Bu)-Asp(*t*-Bu)-$N(CH_3)_2$ 12

After dissolving 0.031 mmol of **6** and 0.039 mmol of HATU in 1 mL of DMF, the reaction mixture was mixed for 10 min. Following the addition of 0.076 mmol of TMP, the reaction mixture was kept for 48 h at room temperature. The product was diluted with 30 mL of ethyl acetate, washed once with 5 mL 1 M HCl and 5 mL of saturated NaCl in water. After drying with sodium sulfate the organic layer was evaporated in vacuo to afford a colorless oil. The product was purified by analytical RP-HPLC and lyophilized. Yield: 4 mg (18%); ^{1}H NMR (600 MHz, DMSO-d_6) $\delta = 8.19$-8.15 (m, Asp-NH, Glu-NH), 7.91 (d, $J = 8.6$ Hz, Ile-NH), 7.44 (d, $J = 8.4$ Hz, Thr-NH), 4.98 (m, Asp-C_α-H), 4.34 (m, Glu-C_α-H), 4.20 (m, Thr-C_α-H), 4.19 (m; Ile-C_α-H), 3.87 (m, Thr-C_β-H), 2.99 (s, N-$CH_3$1), 2.80 (s, N-$CH_3$2), 2.71 (m, Asp-C_β-H1), 2.35 (m, Asp-C_β-H2), 2.20 (m, Glu-Cγ-H_2), 1.86 (m, Glu-C_β-H1), 1.85 (s, Ac-Ile), 1.70 (m, Glu-C_β-H2), 1.69 (m, Ile-C_β-H), 1.41 (m, Ile-C_γ-H1), 1.39-1.37 (m, Asp-*t*-Bu, Glu-*t*-Bu), 1.10 (s, Thr-*t*-Bu), 1.08 (m, Ile-C_γ-H2), 0.98 (d, $J = 6.2$ Hz, Thr-CH_3), 0.82-0.75 (m, Ile-C_β-CH_3, Ile-C_γ-CH_3); ^{13}C NMR (50 MHz, DMSO-d_6) $\delta = 171.7$, 171.3, 170.7, 169.22, 169.19, 169.0, 168.6, 80.0 (C-*t*-Bu), 79.6 (C-*t*-Bu), 73.5 (C-*t*-Bu), 66.7 (C_βH-Thr), 57.3 (C_αH-Thr), 56.6 (C_αH-Ile), 51.7 (CH-Glu), 45.4 (CH-Asp), 37.6 (CH_2-Asp), 36.45 (C_βH-Ile), 36.45 (N-$CH_3$1), 35.2 (N-$CH_3$2), 31.2 ($C_\gamma H_2$-Glu), 27.8 ($3 \times CH_3$-*t*-Bu), 27.7 ($3 \times CH_3$-*t*-Bu), 27.6 ($3 \times CH_3$-*t*-Bu), 26.8 ($C_\beta H_2$-Glu), 24.3 (CH_2-Ile), 22.4 (CH_3-acetyl), 18.9 (CH_3-Thr), 15.3 (C_β-CH_3-Ile), 10.9 (C_γ-CH_3-Ile); HPLC, System 3: $t_R = 41.17$ and 41.97 min; System 5: $t_R = 16.16$ and 16.83 min; Diastereomeric ratio: L/D: 90.66/9.34%; purity: 92.16% at $\lambda = 210$ nm; MS (ESI) m/z calcd for $C_{35}H_{63}N_5O_{10}$: 713.9. Found: m/z 714.5 $(M+H)^+$, 658.4 $(M-(t\text{-Bu})+H)^+$, 602.4 $(M-(2 \times t\text{-Bu})+H)^+$, 546.3 $(M-(3 \times t\text{-Bu})+H)^+$ The structure was also identified by DEPT and 2D NMR (COSY, HSQC) spectra.

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