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Delayed fibril formation of amylin(20–29) by incorporation of alkene dipeptidosulfonamide isosteres obtained by solid phase olefin cross metathesis

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Abstract—The synthesis of a new peptidomimetic structure, the alkene dipeptidosulfonamide isostere, is described. The synthesis is based on a cross metathesis reaction between two allylic building blocks, both in solution and on the solid phase. This method was also applicable to the solid phase synthesis of alkene dipeptide isosteres. Derivatives of amylin(20–29) containing the alkene dipeptidosulfonamide isostere as well as the alkene dipeptide isostere were successfully synthesized using the solid phase cross metathesis method. Investigation of relations between structure and fibril formation of these amylin(20–29) derivatives showed retardation of fibril formation and altered secondary structures, compared to native amylin(20–29).

The replacement of a backbone amide bond in peptides is a strategy which has been widely used to study peptide backbone interactions as well as for stabilization of peptides toward enzymatic degradation. An amide bond surrogate that mimics the geometry of the amide bond (**A**, Fig. 1) is the (*E*)-alkene dipeptide isostere (**B**, Fig. 1).[1]

This isostere has been mainly applied in the synthesis of dipeptide mimics.[1] Only a few papers describe the incorporation of the alkene dipeptide isostere in longer peptides probably partly due to the difficulties involved in the synthesis of the isosteres. Another amide bond surrogate is the sulfonamide bond (**C**), which increases the flexibility of the backbone and is resistant to enzymatic degradation.[2c] Although the sulfonamide-group is a weaker hydrogen bond acceptor, it still can form hydrogen bonds, mainly via the NH, which is a better hydrogen bond donor than an amide NH. The sulfonamide peptidomimetic is conveniently accessible[3] and has been used for incorporation into peptides.[2] A disadvantage of peptidosulfonamides (**C**, Fig. 1) might be the presence of an additional carbon atom in each amino sulfonic acid residue, which is needed for stability reasons.[4] As a result, there is no exact match of a peptidosulfonamide and its parent peptide. We envisioned that by combining the alkene dipeptide isostere with the sulfonamide a new useful peptidomimetic could be designed and synthesized: the alkene dipeptidosulfonamide isostere (**D**, Fig. 1). This isostere has the same backbone length as the parent (di)peptide and also contains a sulfonamide moiety. For the synthesis of the alkene dipeptidosulfonamide isostere it was decided to modify a known cross metathesis procedure for the

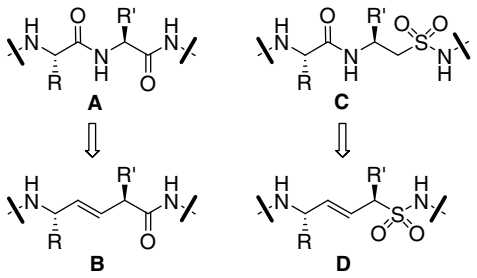

Figure 1. General structures of a dipeptide (**A**), an alkene dipeptide isostere (**B**), a peptide-peptidosulfonamide (**C**), and an alkene dipeptidosulfonamide isostere (**D**).

Keywords: Alkenedipeptidosulfonamide isosteres; Alkenedipeptide isosteres; Peptidomimetics; Solid phase cross metathesis; Amyloid.
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synthesis of alkenedipeptide isosteres.[1j] From the available methods described in the literature, this was the most attractive one because of the relatively few reaction steps, and since its direct applicability to the synthesis of peptides containing a variety of sequences. Several other methods for the synthesis of alkene dipeptide isosteres described in the literature require many, sometimes tedious, reaction steps. In addition, these procedures mostly have been optimized for the synthesis of a specific sequence, and optimization is needed for the synthesis of other sequences.

Besides cross metathesis in solution, we were also interested in application of cross metathesis on the solid phase, since peptides are usually synthesized on a solid support. The advantage of this approach would be that in principle any peptide sequence can be prepared using easily accessible building blocks.

For cross metathesis in solution, the N-terminal building block derived from glycine (**1a**) was prepared by Boc-protection of allylamine. Side chain containing N-terminal building blocks **1b** and **1c** (Scheme 1) were easily prepared from Boc-protected amino acids. Reduction to the amino alcohols was followed by a Swern oxidation to give the corresponding aldehydes.[5–7]

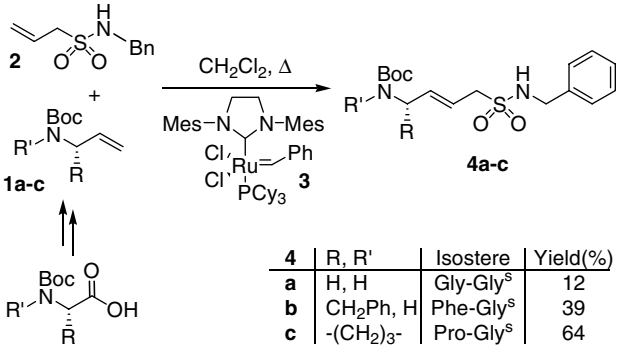
A Wittig olefination with methyl triphenylphosphonium bromide afforded Boc-protected allylic amines derived from phenylalanine (**1b**) and proline (**1c**).[7] For the 'S-'terminal building block, allylsulfonic acid was converted into benzyl sulfonamide **2**.[8] The benzylsulfonamide is stable and is easily detected by UV.

First, cross metathesis was carried out using equimolar amounts of Boc-allylamine (**1a**) and sulfonamide **2** (Scheme 1) with Grubbs second generation catalyst **3**[9] (10%) and dichloromethane as a solvent. This reaction afforded mainly homodimerized Boc-allylamine and only a small amount of product (**4a**). From the literature it is known that the molar ratio of cross metathesis partners is related to their differential reactivities.[1j] When two equivalents of Boc-allylamine (**1a**) were used, isostere **4a** was isolated in 12% yield.[10] We expected that less Boc-allylamine homodimerization would occur using a more sterically hindered allylamine. In line with this assumption, sulfonamide **2** reacted with phenylalanine-derived allylamine **1b**, to give isostere **4b** in 39% yield.[10] When proline-derived allylamine **1c** was used the yield of the cross metathesis product (**4c**) was even higher, that is, 64%.[10] This is in agreement with the literature yields for the synthesis of alkene dipeptide isosteres via cross metathesis.[1j] The *E*/*Z* ratios for the isosteres (**4a**–**c**) were found to be higher than 10:1 (^{1}H NMR analysis) which is also comparable to ratios found in the literature for alkene dipeptide isosteres.[1j] Although it was expected that the yields could be improved by optimizing the molar ratio of the cross metathesis building blocks, it was decided to translate the optimal procedure in solution to application in solid phase synthesis and subsequently to optimize the ratio of the cross metathesis reaction partners.

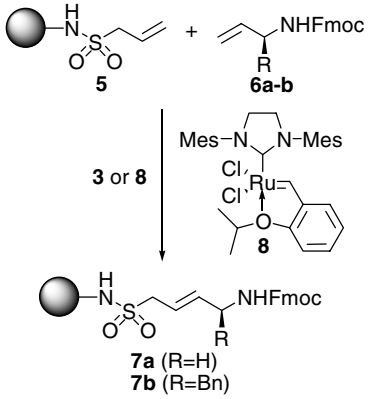
Although solid phase cross metathesis reactions have been hardly described in the literature,[11] it has some advantages compared to cross metathesis in solution, which are reminiscent to solid phase synthesis. The olefin can be added in excess to the resin for driving the reaction to completion, and its non-desired homodimer can be easily removed by filtration. Another possible advantage is less homodimerization of the resin-bound olefin due to its attachment to the solid support.[11a] However, there are reports that the majority of the resin-bound olefin is able to come within reacting distance, leading to homodimerization. Yields reported in the literature vary from low/moderate (11–25%),[11a,b] possibly due to homodimerization, to reasonable/good (54–81%). The latter yields were obtained using simple styrene derivatives with similar reactivities.[11c]

For cross metathesis on the solid phase Fmoc-protected allylamines were chosen to allow determination of the coupling efficiency by measuring the dibenzofulvene-piperidine adduct obtained after cleavage of the Fmoc-group.[12] Fmoc-protected allylamine **6a** was prepared by Fmoc-protection of allylamine,[13] and Fmoc-protected allylamine **6b** (Scheme 2) was prepared from Boc-protected allylamine **1b** by cleavage of the Boc-group followed by introduction of the Fmoc-group using Fmoc-chloride.

Resin **5** was used for optimization of the cross metathesis on the solid phase, which was obtained after coupling



Scheme 1. Synthesis of alkene dipeptidosulfonamide isosteres **4a**–**c** in solution.



Scheme 2. Solid phase synthesis of alkene dipeptidosulfonamide isosteres **7a** and **7b**.

Table 1. Reaction conditions of the optimization of the solid phase synthesis of alkene dipeptidosulfonamides

Entry	**6** R (equiv)		(Ru) %	Lewis acid	Solvent	°C	Time (h)	Loading (%)[b]
1	H	5	**3**, 50		DCM	40	16	17
2	H	10	**3**, 100		DCM	40	16	11
3	H	5	**3**, 50		DCM/MeOH	40	16	0
4	H	5	**8**, 50		DCM/MeOH	40	16	11
5[a]	H	5	**3**, 20		DCM	150[a]	0.5	16
6[a]	H	5	**3**, 20		Toluene	150[a]	0.5	32
7[a]	H	5	**3**, 20	Cy_2BCl	Toluene	150[a]	0.5	21
8	H	5	**3**, 20	Cy_2BCl	Toluene	80	16	19
9	H	5	**8**, 20	Cy_2BCl	Toluene	80	16	35
10	Bn	5	**8**, 20	Cy_2BCl	Toluene	80	16	13
11	Bn	5	**8**, 50	Cy_2BCl	Toluene	80	16	26

[a] Experiment performed in a dedicated microwave reactor.
[b] By spectroscopic determination of the dibenzofulvene piperidine adduct.[12]

of freshly prepared allylsulfonyl chloride to a TentaGel® S NH_2 resin. In the first cross metathesis attempt five equivalents of Fmoc-allylamine (**6a**) and 50% Grubbs catalyst (**3**) were used. After refluxing overnight in dichloromethane the Fmoc-loading of resin **7a** was only 17% of the total available (Table 1, entry 1).

Unexpectedly, doubling the amounts of allylamine **6a** and catalyst gave an even lower loading (11%, entry 2).

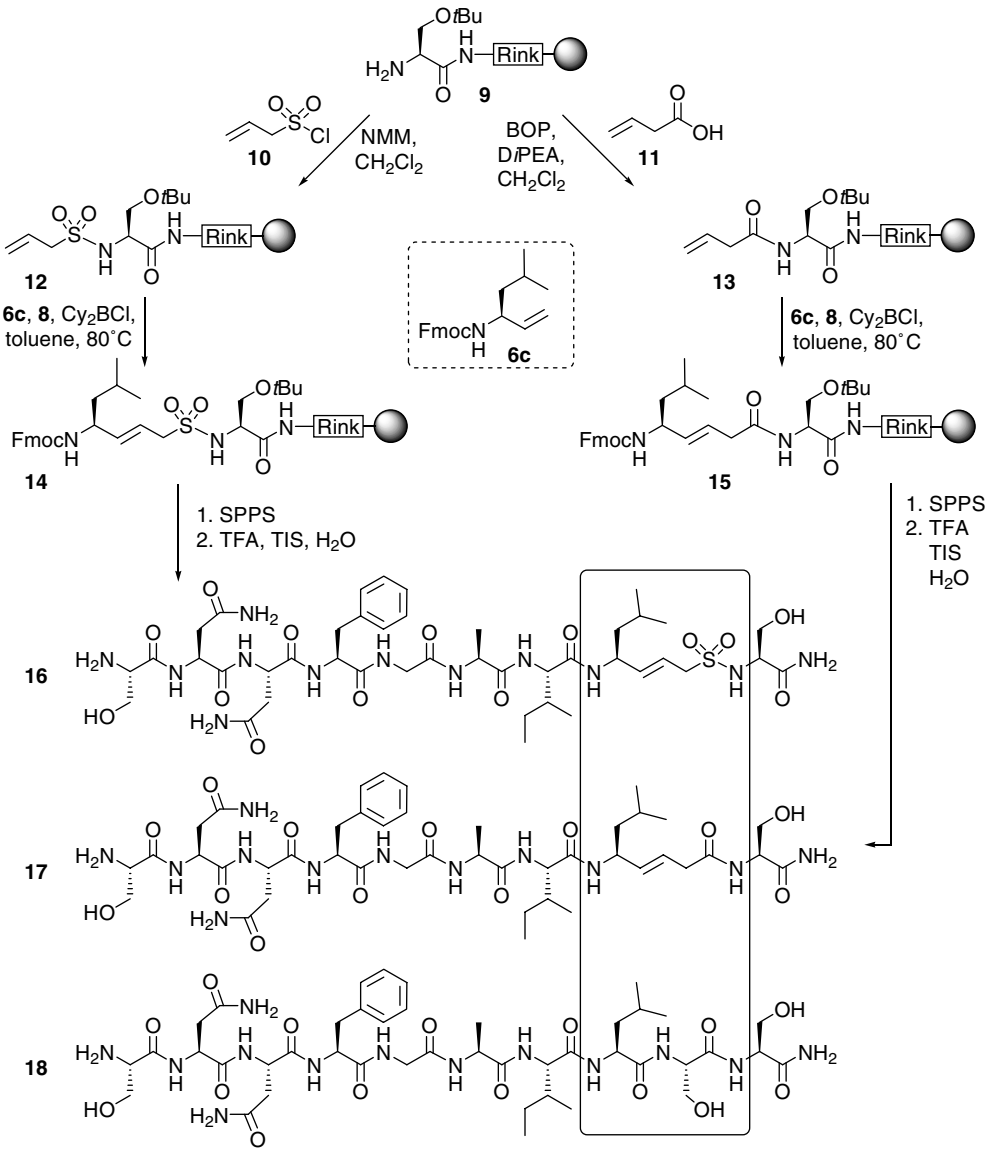
It was observed that the reaction mixture contained a white precipitate, which was found to be the homodimer of allylamine **6a**. When this homodimer precipitates the equilibrium of the reaction will be shifted toward dimerization leading to less cross coupled product **7a**.

When the reaction was performed in a methanol/dichloromethane mixture (1/4, v/v) no precipitate was observed but almost no cross coupled product was found (entry 3) and most of the allylamine (**6a**) had not reacted, probably because of the instability of the catalyst in methanol. The Hoveyda Grubbs catalyst (**8**),[14] which is more stable in polar solvents (Scheme 2), did not lead to a significant increase of Fmoc-loading (11%, entry 4). According to the literature a cross metathesis reaction in solution in the presence of a Lewis acid can increase the yields significantly.[15] Indeed, when 10% Cy_2BCl was used for the synthesis of **7a**, the loading increased to 19% (entry 8) with Grubbs catalyst **3**, and with Hoveyda Grubbs catalyst **8** to 35% (entry 9). Microwave assistance of the cross metathesis reaction did not show significant improvements leading to a loading of 16% (entry 5, 30 min at 150 °C in dichloromethane), 32% (entry 6, in toluene) or 21% (entry 7, in toluene with Lewis acid Cy_2BCl).[16] Using the above optimized conditions for the solid phase cross metathesis obtained with Fmoc-allylamine **6a**, it was assumed that the more sterically hindered Fmoc-allylamine derived from phenylalanine, **6b**, would give a higher efficiency. Unfortunately the obtained loading was only 13% (entry 10), which increased to 26% when the amount of Hoveyda Grubbs catalyst was raised from 20% to 50% (entry 11). Despite these modest results it was decided to apply this optimized cross metathesis procedure (entry 11) to the incorporation of an alkene dipeptidosulfonamide into a peptide.

Amylin(20–29) (**18**, Scheme 3) was chosen for this purpose, since this is a peptide with a high tendency to form (anti)parallel β-sheets, which leads to fibril formation. Investigation of the relations between structure and fibril formation is an important issue in our research. Amylin(20–29) is the highly amyloidogenic region of amylin, also known as human islet amyloid polypeptide, a 37-mer peptide hormone which is involved in the pathogenesis of type II diabetes. So far we have incorporated several peptidomimetic moieties in amylin(20–29) to function as structure breaking entities.[17] Incorporation of the β-peptidosulfonamide moiety resulted in a complete loss of fibril formation.[17c] Instead, supramolecular folding morphologies were observed. Since the alkene dipeptidosulfonamide isostere contains both a sulfonamide and an alkene isostere we were interested in the structural effects upon incorporation in amylin(20–29). In the literature there are only few examples of oligopeptides containing alkene dipeptide isosteres.[1m–r] To our knowledge, the only example in which this isostere was incorporated into a peptide that forms fibrils is the Alzheimer's amyloid peptide (Aβ (1–40)).[1q] This led to a different secondary structure as compared to the native Aβ (1–40) and also spherical aggregates were found instead of fibrils. Based on these results, we were also interested in incorporation of the alkene dipeptide isostere, which can be prepared similarly to the alkene dipeptidosulfonamide isostere by using an allylic carboxylic acid instead of an allylic sulfonic acid. Based on the literature[18] and our previous results,[17a] it was decided to introduce the isosteres at position 27 and 28, occupied by the amino acids leucine and serine. Previously it was found that the serine at position 28 can be replaced by a glycine without losing the aggregation properties of the peptide.[17a]

First, resin bound H-Ser(tBu) (**9**) was reacted with freshly prepared allylsulfonyl chloride (**10**) or with BOP activated 3-butenoic acid (**11**), to yield both sulfonamide **12** and peptide **13** (Scheme 3).

The next step was the cross metathesis reaction with Fmoc-leucine-derived allylamine **6c**, using the above optimized conditions (Table 1, entry 11). The Fmoc-loadings[12] found were 20% for alkene dipeptidosulfonamide isostere **14** and only 10% for alkene



Scheme 3. Solid phase synthesis of amylin(20–29) incorporated with an alkene dipeptidosulfonamide isostere (**16**) and with an alkene dipeptide isostere (**17**). TIS, triisopropylsilane.

dipeptide isostere **15**. However, the latter loading was an unoptimized one. Then, the synthesis was continued using the Fmoc/tBu solid phase peptide synthesis protocols.[19] Peptides **16** and **17** were cleaved from the resin and deprotected using TFA in the presence of triisopropylsilane (TIS) and H_2O as scavengers.[20] Purification using solid phase extraction column chromatography and HPLC afforded alkene dipeptidosulfonamide isostere **16** and alkene dipeptide isostere **17** in 1% and 2% yield, respectively.[21] These yields are comparable to the overall yields described in the literature for the synthesis of peptides containing alkene dipeptide isosteres, which were prepared using preformed alkene dipeptide isostere building blocks. The overall yields of these building blocks vary from 2% to 29%.[1n,o,q] One example describes 11% overall yield for the synthesis of the dipeptide mimic and 36% for the peptide synthesis, leading to an overall yield of 4%.[1n] The syntheses of other peptides using the alkene dipeptide isostere building blocks are reported without yields.[1o,q,r] Our overall yields for the peptide synthesis include the preparation of the dipeptide isostere, which in fact is part of the solid phase peptide synthesis.

Both peptides (**16** and **17**) were characterized by electrospray mass spectrometry.

To study the aggregation behavior, both peptides were dissolved in 0.1% TFA/H_2O to obtain a concentration of 10 mg/mL and rapid gel formation was observed.

Gel formation of sulfonamide isostere **16** was clearly slower than alkene dipeptide isostere **17** (60 vs 30 min), while both were significantly slower than native amylin(20–29) (<10 min)[17a] (**18**). Retardation in fibril formation can be explained by removal of both hydrogen bond donor and acceptor in the peptide backbone. Moreover, in case of the sulfonamide containing isostere

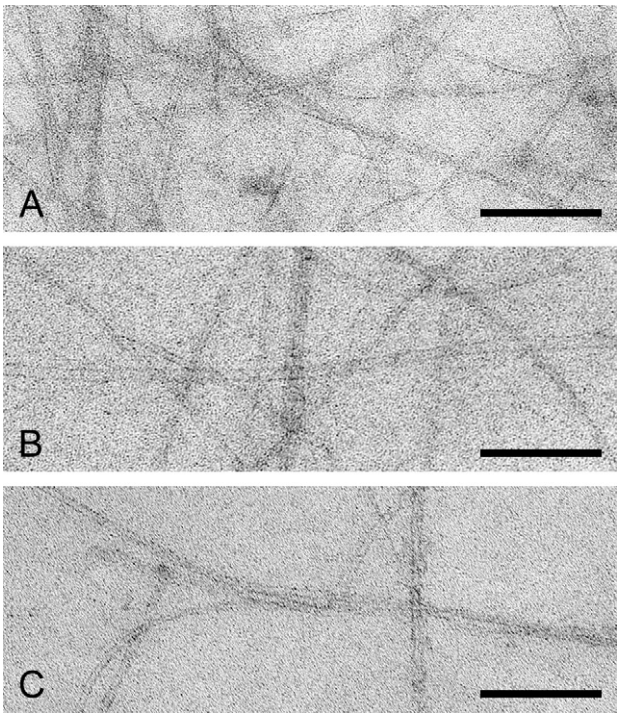


Figure 2. TEM images of amylin(20–29) (A, **18**), amylin(20–29) incorporated with an alkene dipeptide isostere (B, **17**) and with an alkene dipeptidosulfonamide isostere (C, **16**).[22] Scale bars represent 100 nm.

(**16**), the sulfonamide is a weaker hydrogen bond acceptor which is an additional inhibitory factor in the formation of a hydrogen bond network. The presence of amyloid fibrils was confirmed by transmission electron microscopy (TEM) (Fig. 2).

The morphology of both peptide isosteres was similar compared to native amylin(20–29) (**18**). However, the TEM images of peptide isosteres **16** and **17** showed significantly less fibrils than the native amylin(20–29) (**18**). This is in accordance with the delay found in the gel formation of peptide isosteres **16** and **17**. The Fourier transform infrared spectra showed amide I absorptions at 1640 cm^{-1} for both peptides, which indicates either an unordered structure (1640–1648 cm^{-1}) or a β-sheet secondary structure (1625–1640 cm^{-1}).[23]

The absorptions clearly shifted to a higher frequency as compared to native amylin(20–29) (1629 cm^{-1}),[17a] indicating β-sheet secondary structures containing less or weaker hydrogen bonds, which can be ascribed to the presence of a double bond in the isosteres. Unexpectedly, circular dichroism spectroscopy (CD) showed a random coil-like absorption (Fig. 3).

Since fibrils were formed according to the TEM images, a random coil-like secondary structure was not very likely. A combined interpretation of the CD spectra, shifts in the infrared spectra, and TEM images shows that a random coil is not likely as the secondary structure, concluding that a twisted β-sheet is the most probable secondary structure.[24]

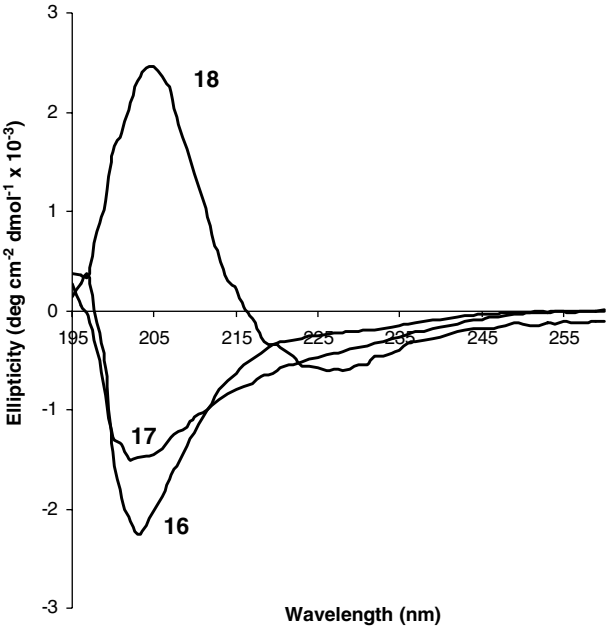


Figure 3. CD spectra of amylin(20–29) (**18**) and amylin(20–29) incorporated with an alkene dipeptidosulfonamide isostere (**16**) and with an alkene dipeptide isostere (**17**). Peptide concentrations: 1 mg/mL in 0.1% TFA/H_2O.

In conclusion, we have developed a method for the preparation of a new peptidomimetic structure, the alkene dipeptidosulfonamide isostere. This isostere was successfully prepared using alkene cross metathesis both in solution and on the solid phase. The yields and conversions mainly depend on the reactivity and steric factors of the alkenes in the cross metathesis reaction in solution. The solid phase synthesis gives overall similar yields as compared to literature yields for the synthesis of peptides containing an alkene dipeptide isostere. An important advantage of the solid phase cross metathesis method described here is that no time-consuming multistep synthesis is required for the preparation of dipeptide peptidomimetic building blocks. The method was found to be also applicable to the solid phase synthesis of alkene dipeptide isosteres. Both isosteres were successfully introduced in the peptide amylin(20–29). Incorporation resulted in retardation of fibril formation for both peptides and altered secondary structures compared to native amylin(20–29) as was confirmed by FTIR and CD.

Acknowledgments

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10. *Synthesis of alkene dipeptidosulfonamide isosteres in solution.* To a degassed (N_2) solution of **1a**–**c** (2.0 mmol) and **2** (1.0 mmol) in CH_2Cl_2 (5 mL, dried on molsieves (4 Å)) was added Grubbs catalyst **3** (42 mg, 50 μmol). The flask was fitted with a reflux condenser and the mixture was refluxed for 16 h while N_2 gas bubbling through. Another portion of catalyst **3** (42 mg, 50 μmol) was added and stirring was continued for 24 h. The mixture was concentrated in vacuo and purified directly using silica gel column chromatography (eluent: gradient EtOAc/hexanes, start: 1/6 → 1/4 → 1/2, v/v). Isosteres **4a**–**c** were obtained as white solids. Characterization data for **4b**: R_f ($MeOH/CH_2Cl_2$, 2/98, v/v): 0.54; ^1H NMR ($CDCl_3$): δ = 1.38 (s, 9H, $C(CH_3)_3$, 2.75 (dd, 1H, $PhCH^a$, J_{vic} = 7.4 Hz, J_{gem} = 13.5 Hz), 2.86 (dd, 1H, $PhCH^b$, J_{vic} = 6.9 Hz, J_{gem} = 13.5 Hz), 3.53 (d, 2H, CH_2SO_2), 4.20 (d, 2H, NCH_2Ph), 4.29 (m, 1H, $CHCH_2Ph$), 4.71 (d, 1H, NHBoc), 5.09 (m, 1H, NHBn), 5.48 (m, 2H, CH=CH), 7.13–7.36 (m, 10H, 2 × Ph); 13C NMR ($CDCl_3$): δ = 28.2 ($C(CH_3)_3$), 40.8 ($CHCH_2Ph$), 47.2 (NCH_2PH), 53.2 ($CHCH_2Ph$), 55.6 (CH_2SO_2), 79.8 ($C(CH_3)_3$), 118.6 ($NCHCH=$), 126.7, 128.0, 128.1, 128.5, 128.7, 129.4, 136.9, 137.0 (Ar-C), 139.5 ($=CHCH_2SO_2$), 155.2 (C=O (Boc)); ESI-MS: calculated for $C_{23}H_{30}N_2O_4S$-Na $[M+Na]^+$: 453.18, found: 453.55.
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20. *Peptide synthesis.* Peptide isosteres **16** and **17** were synthesized on a 0.26 mmol scale on Tentagel Fmoc-Rink-Amide resin in order to obtain C-terminally amidated peptides. Each synthetic cycle consisted of N^{α}-Fmoc removal by a 1 h treatment with 20% piperidine in NMP, washings with NMP (2 × 2 min) and CH_2Cl_2 (5 × 2 min), a 45-min coupling step with preactivated Fmoc-amino acid (1.04 mmol), and washings with NMP (2 × 2 min) and CH_2Cl_2 (5 × 2 min). N^{α}-Fmoc amino acids were activated in situ with HBTU/HOBt (1.04 mmol, 0.21 M in NMP) in the presence of D*i*PEA (2.08 mmol). 3-Butenoic acid was coupled using normal amino acid coupling conditions for 3 h. Allylsulfonyl chloride (1.04 mmol), freshly prepared from the corresponding sodium sulfonate using phosgene (purification: silicagel plug, CH_2Cl_2; 85% yield; clear colorless oil),[3] was coupled using CH_2Cl_2 and D*i*PEA, followed by normal washings. The cross metathesis reaction was performed using allyl amine **6c** (1.3 mmol), Hoveyda Grubbs catalyst (**8**, 0.13 mmol), and Cy_2BCl (26 μmol) in toluene (20 mL, degassed (N_2)). After stirring overnight at 80 °C, the resin was washed with methanol (1 × 2 min), NMP (2 × 2 min), and CH_2Cl_2 (5 × 2 min). The peptides were detached from the resin and

deprotected by treatment with TFA/H_2O/TIS (95/2.5/2.5, v/v/v) for 3h. The peptides were precipitated with MTBE/hexane (1/1, v/v) at −20 °C, dissolved in H_2O, and separated from brown ruthenium impurities by a SPE column (C4, eluent = H_2O) and finally lyophilized.

21. *Purification and analysis.* The peptides were purified by dissolving the crude material in a minimal amount of buffer A and loaded onto an Adsorbosphere XL C8 HPLC column (90 Å pore size, 10 μm particle size, 2.2 × 25 cm). The peptides were eluted with a flow rate of 10 mL/min using a linear gradient of buffer B (50% in 40 min) from 100% buffer A (buffer A: 0.1% TFA in CH_3CN/H_2O, 5/95, v/v; buffer B: 0.1% TFA in CH_3CN/H_2O, 95/5, v/v). The purity was determined by analytical HPLC on an Adsorbosphere XL C8 column (90 Å pore size, 5 μm particle size, 0.46 × 25 cm) at a flow rate of 1.0 mL/min using a linear gradient of buffer B (100% in 20 min) from 100% buffer A. Peptides **16** and **17** were characterized by mass spectrometry. Yields: **16**, 1% (2 mg); **17**, 2% (5 mg). ESI-MS mass found (calcd) $[M+H]^+$: **16**, 961.78 (961.51); **17**, 997.40 (997.48). HPLC analysis t_R: **16**, 16.90 min; **17**, 16.78 min. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Shimadzu LC MS QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode.
22. *TEM sample preparation.* Peptide isosteres were dissolved in 0.1% TFA/H_2O to obtain a concentration of 10 mg/mL. These solutions were stored at 4 °C during 3 weeks. The obtained gels were used for TEM measurements.
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