Journal of Peptide Science
J. Peptide Sci. **8**: 307–313 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.404

Synthesis of a 2-Arylsulphonylated Tryptophan: The Antibacterial Activity of Bovine Lactoferricin Peptides Containing Trp(2-Pmc)

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Received 22 March 2002
Accepted 13 April 2002

Abstract: A modified tryptophan, β-[2-(2,2,5,7,8-pentamethylchroman-6-sulphonyl)-indol-3-yl]alanine, Trp(2 − Pmc) = Tpc has been synthesized. Replacement of tryptophan in a bovine lactoferricin model peptide with the modified tryptophan resulted in peptides with a substantially increased antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The most active peptides against each bacterial strain displayed minimal inhibitory concentrations of 7.5 μg/ml. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: tryptophan; side-reaction of tryptophan; Pmc; antibacterial peptide; bovine lactoferricin

INTRODUCTION

As a model for structure-activity relationship studies of antibacterial peptides [1–5], we have used the peptide LFB, FKCRRWQWRMKKLGA, which encompasses residues 17–31 of bovine lactoferrin [6]. In the course of our experiments it was observed that by-products are formed during the acidic cleavage of protecting groups, and that these by-products display a higher antibacterial activity than the desired peptides. The side-chains of the Arg residues were protected during peptide synthesis by the 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) protecting group, and MS analyses revealed that the highly active by-products contain a Pmc-moiety. NMR studies of the by-products further revealed that they contained a Trp(Pmc) residue, rather than a protected Arg residue (M. L. Skar, unpublished results). This transfer of a Pmc moiety from Arg to Trp is a known side-reaction in peptide synthesis [7,8]. Several other side-reactions are reported to occur on the indole side-chain of Trp during the final TFA mediated deprotection and cleavage steps in solid phase peptide synthesis (for a review see [9]). The indole side-chain can also undergo sulphanylation and sulphonylation reactions with electrophilic species generated from *N*-terminal [10] and other Arg side-chain protecting groups [7,11,12], respectively, resulting in the formation of 2-substituted Trp residues (Scheme 1). The amount of Pmc-modified Trp produced is reported to be close to 100% in TFA when only one amino acid separates the Trp and Arg residues [13] whereas intermolecular transfer of Pmc from Arg to Trp only yields 68% of the modified peptide [13]. Riniker and Hartmann [8] have isolated a Trp(2-Pmc) derivative in 45% yield

Abbreviations: LFB, bovine lactoferricin residues 17–31; MIC, minimal inhibitory concentration; Pbf, 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulphonyl; Tpc, β-[2-(Pmc)-indol-3-yl]alanine, i.e. Trp(2-Pmc).

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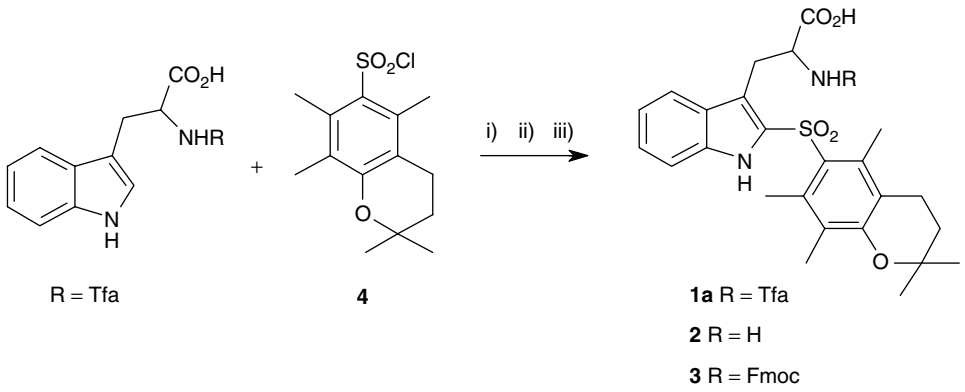
Contract/grant sponsor: Norwegian Research Council; Contract/grant number: HS2037/611501.
Contract/grant sponsor: Alpharma A/S (Norway).



Scheme 1 *Reagents and conditions*: (i) TFA, −12 °C, 24 h, 86%; (ii) **1a**, LiOH, THF:MeOH:H_2O, 2 : 2 : 1, r.t., 24 h, 94%; (iii) **2**, Fmoc-OSu, $NaHCO_3$, DME/H_2O, 2 days, 74%.

by stirring Fmoc-Arg(Pmc)-OH and Ac-Trp-OH in TFA. Minor amounts of the 5- and 6-isomers were also reported. Due to the effect of the Trp(2-Pmc) residue in antibacterial peptides and the desire to prepare peptides with this novel residue in defined positions, a method was developed for the synthesis of Fmoc-Trp(2-Pmc)-OH = Fmoc-Tpc-OH (Scheme 1), and used to incorporate Tpc residues into the model peptide LFB as a Trp replacement at positions 6 and/or 8; the peptides obtained were tested for antibacterial activity against the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus*.

MATERIALS AND METHODS

All the commercially available reagents were used without further purification. 2,2,5,7,8-Pentamethylchromane and 2,2,5,7,8-pentamethylchroman-6-sulphonyl chloride PmcCl, **4**, were prepared as described by Ramage *et al.* [14], 2-phenylethylguanidine and its Pmc derivative were prepared as described by Eggleston [15] and Tfa-Trp-OH was prepared as described by Curphey [16].

Peptide Synthesis

Peptides were synthesized, purified and analysed as previously reported [3], except that Pbf was used to protect the side chains of Arg, and Boc was used to protect the unmodified Trp side chains. The purity of all peptides was found to be >96%. The antibacterial assay was as described previously [3], using the following concentration series: 50, 35, 25, 20, 15, 10, 7.5, 5, 2.5 and 1.0 μg/ml.

N^{α}-(2,2,2-trifluoro-acetyl)-β-(2-(2,2,5,7,8-pentamethylchroman-6-sulphonyl)-indol-3-yl)alanine, Tfa-Trp(2-Pmc)-OH = Tfa-Tpc-OH (1a)

Tfa-Trp-OH (1.141 g, 3.80 mmol) and **4** (0.460 g, 1.519 mmol) were added to a dried and cooled 50 ml round bottomed flask. The flask was purged with argon and cooled to −12 °C. TFA (15 ml) was cooled to -12 °C and was transferred by cannula to the flask. The resulting light yellow mixture was stirred at -12 °C for 24 h. The reaction mixture was diluted with 25 ml chloroform and 25 ml water while cold, and the layers were separated. The aqueous phase was extracted with 2 × 25 ml chloroform, the combined organic layers were washed with 25 ml aqueous NaCl and evaporated to yield 0.91 g of a white solid. The crude product was submitted to flash column chromatography ($CHCl_3$–MeOH–HOAc, 95 : 4 : 1, $R_f = 0.24$). Fractions containing the product were pooled and extracted with 3 × 100 ml aqueous NaCl. Evaporation of the organic phase afforded the desired compound (0.740 g, 86%) as a white foamy solid. Mp = 110–112 °C (dec); Found: 566.1722. $C_{27}H_{29}F_3N_2O_6S$ (M^+) requires 566.1698; $[\alpha]_D^{22} + 5.37$ (c 1.01, $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 9.45 (1H, s, indole NH), 8.81 (1H, br s, CO_2H), 8.26 (1H, d, $J = 5.9$, C(2)N*H*), 7.66 (1H, d, $J = 8.1$, indole C*H*), 7.44 (1H, d, J = 8.4, indole C*H*), 7.36–7.32 (1H, m, indole C*H*), 7.21–7.18 (1H, m, indole C*H*), 4.60–4.54 (1H, m, C(2)*H*), 3.31 (1H, dd, $J = 14.7$, 4.0, C(3)*H*), 3.06 (1H, dd, $J = 14.7$, 11.0, C(3)*H′*), 2.60 (2H, t, $J = 7.0$, Pmc CH_2), 2.46 (3H, s, Pmc-CH_3), 2.41 (3H, s, Pmc-CH_3), 2.07 (3H, Pmc-CH_3), 1.81 (2H, t, $J = 7.0$, Pmc CH_2), 1.31 (6H, s, Pmc C(2)(CH_3)$_2$); δ_C (100 MHz; $CDCl_3$) 175.03, 158.04 (q, $^2J_{CF} = 38.4$), 156.27,

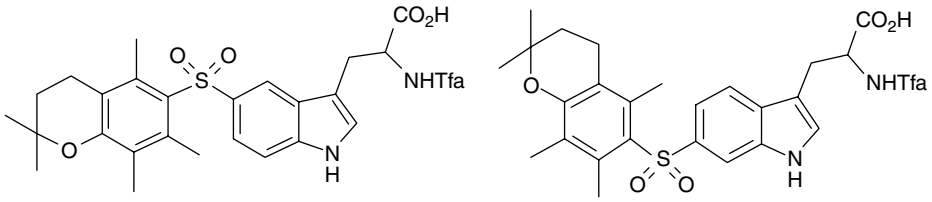


Figure 1 Minor isomers from the arylsulphonylation of Tfa-Trp-OH.

137.54, 137.36, 135.36, 134.88, 128,46, 126.83, 126.22, 125.62, 121.74, 119.98, 119.33, 115.58 (q, $^1J_{CF} = 287.5$), 112.75, 112.60, 74.76, 53.48, 32.41, 26.77, 26.70, 25.21, 21.31, 17.77, 16.90, 12.14.

Analytical data for Tfa-Trp(Pmc)-OH isomers. **1b:** Found: 567.3, 589.2 (M + Na). $C_{27}H_{30}F_3N_2O_6S$, (MH^+) requires 567.61; δ_H (400 MHz; $CDCl_3$) 9.27 (1H, s), 7.87 (1H, s), 7.49 (1H, d, $J = 8.8$), 7.33 (1H, d, $J = 8.8$), 7.30–7.26 (1H, m), 7.02 (1H, s), 4.78–4.67 (1H, m), 3.30–3.20 (2H, m), 2.55 (2H, t, $J = 6.6$), 2.41 (6H, s), 2.04 (3H, s), 1.77 (2H, t, $J = 6.6$), 1.28 (6H, s); δ_C (100 MHz; $CDCl_3$) 174.11, 157.11 (q, $^2J_{CF} = 38.4$), 155.53, 137.58, 137.52, 137.38, 134.71, 130.28, 128.64, 127.54, 125.02, 118.93, 118.81, 116.58, 115.58 (q, $^1J_{CF} = 287.5$), 110.22, 109.36, 74.32, 53.74, 32.59, 26.80, 26.58, 21.32, 18.39, 17.38, 12.11. **1c**: Found: 567.3, 589.2 (M + Na). $C_{27}H_{30}F_3N_2O_6S$, (MH^+) requires 567.61; δ_H (400 MHz; $CDCl_3$) 8.89 (1H, d, $J = 2.0$), 8.31 (1H, s), 7.75 (1H, d, $J = 8.4$), 7.33–7.26 (2H, m), 7.12 (1H, d, $J = 2.0$), 4.94–4.89 (1H, m), 3.35 (1H, dd, $J = 14.8$, 4.9), 3.26 (1H, dd, $J = 14.8$, 6.8), 2.61 (2H, t, $J = 6.8$), 2.45 (3H, s), 2.44 (3H, s), 2.09 (3H, s), 1.80 (2H, t, $J = 6.8$), 1.31 (6H, s); δ_C (100 MHz; $CDCl_3$) 173.02, 156.91 (q, $^2J_{CF} = 38.4$), 155.54, 137.66, 137.64, 137.49, 135.59, 128.53, 127.01, 126.05, 125.02, 119.40, 118.82, 117.51, 115.63(q, $^1J_{CF} = 287.2$), 111.98, 110.78, 74.34, 53.19, 32.63, 27.08, 26.84, 26.81, 21.36, 18.42, 17.39, 12.13.

Analytical data for Tfa-Trp-OH dimers. **1d:** Found 600.3. $C_{26}H_{22}F_6N_4O_6$ (M^+) requires 600.48; R_t = 17.4 min. (RP-HPLC); δ_H (400 MHz, methanol-d_4) 7.56 (1H, d, $J = 7.9$), 7.34 (1H, d, $J = 7.4$), 7.29–7.26 (1H, m), 7.26–7.21 (1H, m), 7.11–7.06 (1H, m), 7.05–7.02 (1H, m), 7.01–6.99 (1H, m), 6.94 (1H, d, $J = 7.7$), 5.11 (1H, d, $J = 7.6$), 4.82–4.78 (2H, m), 3.77–3.72 (1H, m), 3.56 (1H, dd, J = 14.8, 5.7), 3.35–3.32 (1H, m), 2.55–2.48 (1H, m), 2.32–2.25 (1H, m). **1e**: Found 600.3. $C_{26}H_{22}F_6N_4O_6$ (M^+) requires 600.48; $R_t = 16.6$ min. (RP-HPLC); δ_H (400 MHz; methanol-d_4) 7.55 (1H, d, $J = 7.9$), 7.30 (1H, d, $J = 7.4$), 7.27 (1H, d, $J = 8.1$), 7.25–7.20 (1H, m), 7.10–7.06 (1H, m), 7.05–7.02 (1H, m), 7.02–6.98 (1H, m), 6.92 (1H, d, $J = 7.8$), 5.09–5.04 (1H, bs), 4.76–4.70 (1H, m), 4.42–4.38 (1H, m), 3.86–3.79 (1H, m), 3.52–3.46 (1H, m), 3.25–3.18 (1H, m), 2.58–2.50 (1H, m), 2.38–2.29 (1H, m).

β-(2-(2,2,5,7,8-pentamethylchroman-6-sulphonyl)-indol-3-yl)alanine, H-Tpc-OH, (2)

To a stirred solution of **1a** (0.77g, 1.36 mmol) in THF-MeOH-H_2O (2 : 2 : 1) (35 ml) was added LiOH monohydrate (0.31 g, 7.39 mmol) and the mixture stirred at room temperature for 24 h. The reaction mixture was evaporated *in vacuo* to a small volume, and acidified to pH 2–3 by careful addition of 1M HCl. After cooling, the precipitate was isolated by filtration and dried *in vacuo* to afford the desired compound (0.65 g, 94%) as a white powder. Mp = 178–181 °C (dec); Found: 471.1940. $C_{25}H_{31}N_2O_5S$ (MH^+) requires 471.1948; $[\alpha]_D{}^{25} - 1.62$ (*c* 1.05, methanol); δ_H (400 MHz; methanol-d_4) 7.70 (1H, d, $J = 8.2$, indole C*H*), 7.49 (1H, d, $J = 8.4$, indole C*H*), 7.34–7.30 (1H, m, indole C*H*), 7.19–7.15 (1H, m, indole C*H*), 3.91 (1H, dd, $J = 9.9$, 5.5, C(2)*H*), 3.25 (1H, dd, $J = 14.9$, 5.5, C(3)*H*), 3.17 (1H, dd, $J = 14.9$, 9.9, C(3)*H'*), 2.65 (2H, t, $J = 6.8$, Pmc CH_2), 2.44 (3H, s, Pmc-CH_3), 2.38 (3H, s, Pmc-CH_3), 2.07 (3H, s, Pmc-CH_3), 1.83 (2H, t, $J = 6.8$, Pmc-CH_2), 1.32 (6H, s, Pmc-C(2)(CH_3)$_2$); δ_C (100 MHz; methanol-d_4) 170.19, 155.68, 137.52, 137.13, 135.55, 135.54, 129.09, 127.28, 125.21, 124.92, 120.89, 119.39, 119.27, 112.48, 110.98, 74.45, 53.66, 32.13, 25.61, 25.60, 25.07, 20.78, 16.65, 15.78, 10.77.

Fmoc-(β-(2-(2,2,5,7,8-pentamethylchroman-6-sulphonyl)-indol-3-yl)alanine, Fmoc-Tpc-OH (3)

A stirred suspension of **2** (0.522 g, 1.03 mmol) in 5 ml DME was diluted with 2 ml water, and $NaHCO_3$ (0.175 g, 2.08 mmol) was added in small portions. The resulting mixture was stirred for 10 min before

Fmoc-OSu (0.373 g, 1.08 mmol) was added, and the 'milky' reaction mixture was stirred at room temperature for 2 days. The resulting clear yellow solution was evaporated to a small volume, acidified to pH 1 with 5M HCl and extracted 3 × 10 ml EtOAc. The combined organic layers were washed with saturated NaCl, dried over $MgSO_4$ and evaporated to give a yellow solid (0.78 g). Flash column chromatography (CH_2Cl_2-MeOH, 92.5:7.5, $R_f = 0.21$) afforded the product (0.525 g, 74%) as a white solid. Mp = 142–143 °C (dec); Found: 693.2614. $C_{40}H_{41}N_2O_7S$ (MH^+) requires 693.2629; $[\alpha]_D^{22} - 13.05$ (*c* 1.07, $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 9.49 (1H, s, indole N*H*), 7.72 (3H, *J* = 7.7, Fmoc C*H* and indole C*H*), 7.57 (2H, d, *J* = 7.3, Fmoc C*H*), 7.41 (1H, d, *J* = 8.1, indole C*H*), 7.37–7.33 (2H, m, Fmoc C*H*), 7.29–7.20 (3H, m, Fmoc C*H* and indole C*H*), 7.15 (1H, t, *J* = 7.3, indole C*H*), 6.52 (1H, d, *J* = 5.1, C(2)N*H*), 4.50–4.42 (1H, m, C(2)*H*), 4.27–4.20 (1H, m, Fmoc C*H*-CH_2-O), 4.19–4.10 (2H, m, Fmoc CH-CH_2-O), 3.28–3.20(1H, m, C(3)*H*), 3.08–3.00 (1H, m, C(3)*H'*), 2.55 (2H, t, *J* = 6.2, Pmc-CH_2), 2.49 (3H, s, Pmc-CH_3), 2.45 (3H, s, Pmc-CH_3), 2.05 (3H, s, Pmc-CH_3), 1.73 (2H, t, *J* = 6.2, Pmc-CH_2), 1.26 (6H, s, Pmc C(2)(CH_3)$_2$); δ_C (100 MHz; $CDCl_3$) 176.55, 156.62, 156.08, 143.97, 143.91, 141.26, 141.25, 137.59, 137.44, 134.92, 128.78, 127.69, 127.66, 127.22, 127.16, 125.90, 125.61, 125.57, 125.49, 121.44, 120.43, 119.88, 119.23, 112.60, 77.37, 74.60, 67.35, 54.47, 47.02, 32.44, 26.79, 26.71, 21.33, 17.81, 16.94, 12.19.

RESULTS AND DISCUSSION

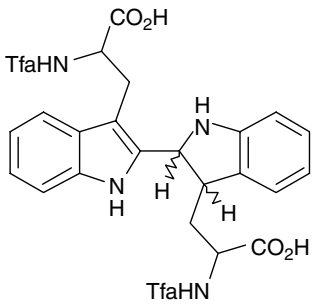
Amino Acid Synthesis

The introduction of the Pmc group onto the Trp side chain was initially attempted by stirring Fmoc-Trp-OH with the Pmc derivative of 2-phenylethyl guanidine [15] in neat TFA. A screening of the reaction conditions using a fractional factorial design was undertaken using three variables at two levels. The temperature was set to 19 °C or 50 °C, the ratio between Fmoc-Trp-OH and the Pmc protected guanidine was set to either 2 or 5, whereas the concentration was set to 0.05M or 0.10M with respect to the sulphonylating agent. The screening experiments revealed that a large excess of Fmoc-Trp-OH was preferred, and due to the tedious workup of the reaction mixture no more than ~25% of the desired product could be isolated. The use of Ac-Trp-OH, enabled the isolation of the desired Pmc-modified isomer by flash column chromatography, but removal of the acetyl group proved to be difficult. However, by using Tfa-Trp-OH (Scheme 1), the three isomeric products formed [17] (minor isomers shown in Figure 1) could be separated by flash column chromatography and the Tfa-protecting group was readily removed by treatment with LiOH in THF–MeOH–H_2O (2:1:1), to give **2** in 94% yield. In these preliminary experiments the Pmc group was introduced by transfer from the Pmc derivative of 2-phenylethyl guanidine. However, this proved to be unnecessary as the chloride **4** works equally well as the electrophilic species in the sulphonylation reaction. At room temperature it was found that a longer reaction time gave less of the desired regioisomer, and **1a** was also shown by RP-HPLC analyses to isomerize in neat TFA. When 1.5 equivalents of **4** were used at −12 °C for 24 h, less of the unwanted isomers were formed. This simplified the chromatographic separation of the isomers considerably, and allowed isolation of the product in a 53% yield. At room temperature the isolated yield of **1a** varied between 45% and 55%, but required several runs of flash column chromatography. Despite the improved regioselectivity on changing to −12 °C, the yield of **1a** was not increased, as the dimerization (Figure 2) could not be suppressed [18,19–21]. Thus, the extent of dimerization was more or less the same, regardless of what temperature the reactions were run at, and to overcome this, sulphonylation was attempted in glacial acetic acid. No dimerization was reported by Scoffone *et al.* [22] who sulphanylated Trp in glacial acetic acid with nitrophenylsulphanyl chlorides in 70%–80% yield. However, in our case sulphonylation in glacial acetic acid only afforded at best trace amounts of the desired product after 24 h at room temperature. The unwanted dimerization is apparently unavoidable. During SPPS it is recommended to use Boc-protected Trp both to avoid sulphonylation of Trp [12] and to prevent dimerization.

When a 2.5 fold excess of Tfa-Trp-OH was stirred with **4** (Scheme 1) in neat TFA at −12 °C for 24 h, **1a** was isolated in a 86% yield. By extracting the aqueous phase with chloroform, the dimers were retained, and thus did not cause any problems in the chromatographic purification step. Fmoc-protection of **2** by using Fmoc-OSu in DME and water with $NaHCO_3$ as the base allowed the isolation of **3** as a white solid in 74% yield.

Antibacterial Activity of LFB Peptides

The antibacterial activities of the LFB derivatives containing the novel Tpc amino acid are presented



1d, 1e

Figure 2 Dimers of Tfa-Trp-OH.

Table 1 Antibacterial Activity of LFB Peptides Containing Tpc

Peptide[a] name	Molecular[b] mass	MIC[c] *E. coli*	MIC[c] *S. aureus*
LFB	2064.8 (2064.5)	50 (24)	100 (48)
[Tpc^6]-LFB	2331.2 (2331.4)	7.5 (3.2)	10 (4.3)
[Tpc^8]-LFB	2331.2 (2331.4)	15 (6.4)	7.5 (3.2)
[Tpc^{6,8}]-LFB	2597.5 (2597.5)	25 (9.6)	7.5 (2.9)

[a] Sequence of LFB: FKCRRW^{6}QW^{8}RMKKLGA.
[b] Molecular mass calculated and (observed) including Acm-protected cysteine.
[c] Minimal inhibitory concentration in μg/ml and (μM)

in Table 1. As was observed for the original peptide by-products containing a Pmc moiety, the replacement of the Trp residues by Tpc resulted in a major increase in antibacterial activity against both *E. coli* and *S. aureus*. LFB derivatives containing the large aromatic Tpc were more than 13 times as active as LFB against *S. aureus*, whereas the activity against *E. coli* was increased more than 6-fold.

The influence of the size of aromatic residues on the antibacterial activity of LFB derivatives has been the subject of considerable research in our group [3,4]. Replacement of one of the two Trp residues in LFB by Ala diminishes antibacterial activity [2], whereas the replacement of Trp by larger unnatural residues leads to more active peptides [3,4]. LFB is believed to be antibacterial by a membrane active mechanism, in which lipophilic residues are inserted into the bacterial cell membrane, thereby disturbing the packing of the phospholipids. Upon interaction of the core peptide of LFB, RRWQWR-NH_2, with SDS-micelles, the two Trp residues are located deeper into the micelles than are the other residues [23]. It has also been suggested that Trp residues function as membrane 'anchors' for membrane proteins [24,25]. With its larger side chain, Tpc can disturb the packing of the phospholipids more efficiently than Trp itself, resulting in enhanced antibacterial activity. The side chain volume of Tpc was calculated as described previously [4] and found to be 365 Å^3. It is the largest aromatic amino acid we have employed in our studies, and Tpc-containing LFB derivatives displayed MIC values in the same range as the most active peptides previously reported by us [4]. It was noteworthy that the replacement of both Trp residues in LFB by Tpc did not increase the antibacterial activity relative to peptides containing one Tpc residue. However, we have also previously found that the introduction of two very large aromatic residues into LFB can be unfavourable, especially against *E. coli*. These observations may be a result of the peptides becoming too hydrophobic, which is expected to render them less effective in associating with the bacterial cell membranes. The introduction of two large residues in positions 6 and 8 may also result in folding of the peptide into a conformation that is less efficient for disruption of bacterial cell membranes. A similar effect has also been observed when additional Trp residues have been incorporated into LFB; it was found that an LFB peptide containing five Trp residues was less antibacterial than LFB peptides containing four Trp residues [5]. Although the replacement of Trp by Tpc boosted the antibacterial activity more against *S. aureus*, [Tpc^6]-LFB is one of the most active peptides against *E. coli* of the LFB derivatives we have prepared. It was also noteworthy that [Tpc^6]-LFB and [Tpc^8]-LFB showed MIC values of 7.5 μg/ml against *E. coli* and *S. aureus*, respectively. In contrast, when other large aromatic residues have been incorporated into LFB, such low MIC values have only been obtained against one or the other of these bacterial strains [4]. This underscores the importance of having a vast diversity of residues to choose from in the preparation of novel highly active antibacterial peptides.

CONCLUSION

Tfa-Trp-OH can be directly arylsulphonylated at position 2 by **4** in neat TFA, and the product

can be converted to Fmoc-Tpc-OH **3**, use of which to introduce Tpc residues into our model peptide LFB as Trp replacements results in an up to 13-fold increased ability to inhibit bacterial growth. As we have previously reported, the increase in antibacterial activity can be correlated with the molecular volume of the Tpc side-chain. The method we have established for the synthesis of Fmoc-Tpc-OH **3** provides an important novel building block and opens up new avenues for the exploration of the structure–activity relationships of antibacterial peptides.

Acknowledgements

This work was supported by the Norwegian Research Council (grant number HS2037/611501) and Alpharma A/S (Norway). We thank Tore Lejon at the Department of Chemistry for designing the screening experiments. We thank Gerd Berge at the Department of Biochemistry for skilful peptide synthesis. We thank Lars Vorland and Ørjan Samuelsen at the University Hospital of Tromsø for providing us with the biological data. We also thank Morten B. Strøm for valuable discussions and suggestions in writing this manuscript.

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17. For analytical data of the minor isomers, 1b and 1c see materials and methods.
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