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Protection of the indole nucleus of tryptophan in solid-phase peptide synthesis with a dipeptide that can be cleaved rapidly at physiological pH

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ABSTRACT

The synthesis of a new derivative of tryptophan Fmoc-Trp(Boc-Sar-Sar)-OH is described. Fmoc-Trp(Boc-Sar-Sar)-OH is introduced into peptides by solid-phase peptide synthesis and during treatment with TFA at the end of the synthesis, the Boc group is cleaved and the peptide is obtained with the indole nucleus modified with the sarcosinyl–sarcosinyl (Sar-Sar) moiety. This modification will introduce a cationic charge that improves the solubility of the peptide during HPLC purification. The Sar-Sar moiety is cleaved upon exposure to physiological pH. The Boc-Sar-Sar group might, therefore, also find use in the design of pro-drugs of indole-containing compounds.

Protecting groups on the side chains of amino acids are necessary to avoid side-reactions both during assembly of the peptide chain and cleavage of the peptide from the resin in solid-phase peptide synthesis. The protecting groups available today are, in most cases, associated with relatively few side-reactions so it would appear that no need for new side-chain protecting groups except for some special cases such as the β-carboxylic group on aspartic acid residues in sensitive sequences.[1] However, in the majority of cases the peptide has to be purified after cleavage from the resin and for many peptides the purification step is more difficult and time-consuming than synthesis of the crude peptide itself. The major reasons for this are the problems associated with poor solubility. Many peptides are hydrophobic and/or prone to β-sheet formation and can, therefore, be difficult to dissolve in sufficient amounts to be loaded onto preparative HPLC columns.[2] Moreover, the elution profiles of such peptides often show broad peaks with low resolution and the isolated yields are usually low.[3] Strategies to circumvent these problems have been suggested. Choma and co-workers have shown that if hydrophobic peptides are synthesized with a 'solubilising tail', a C-terminal extension of the peptide with several cationic amino acid residues linked to the peptide with an ester bond, the solubility is increased and HPLC purification can be facilitated.[4] However, after purification of the peptide the ester bond in the 'solubilising tail' has to be cleaved by relatively strong alkali.

In two recent publications we have addressed these problems and have presented the solubilising protecting groups for the side-chains of tyrosine and tryptophan.[5,6] These protective groups are stable during the synthesis of the peptide but after cleavage of the peptide from the resin, an amino group is exposed which increases the solubility of the peptide in the acidic solvents used for HPLC. After purification of the peptide, the protecting groups are removed by an intramolecular cyclization reaction at slightly alkaline pH. In one of these studies we showed that if the indole nucleus of tryptophan is protected during the synthesis with a Boc-4-(*N*-methylamino)butanoyl group, tryptophan is protected against the modification during cleavage of the peptide from the resin with TFA and the cationic 4-(*N*-methylamino)butanoyl group remains on the peptide during the purification. Cleavage of the 4-(*N*-methylamino)butanoyl group after purification of the peptide proceeds via an intramolecular cyclization but this reaction required relatively high pH and the half-life was 5–7 min at pH 9.5. It would be expected that most peptides would not be modified at that pH, but in general it is desirable to keep the pH low, as base-catalysed side reactions such as aspartimide formation at some asparagine sequences are known to be relatively fast in alkaline aqueous solutions.[7]

A large proportion of all synthetic peptides are used in various biological experiments at physiological pH. Dissolving the peptides prior to the experiment can be a major problem especially if high concentrations of organic solvents are to be avoided. Most peptides used in biological experiments are obtained as TFA salts from lyophilized HPLC fractions and increasing the number of amino

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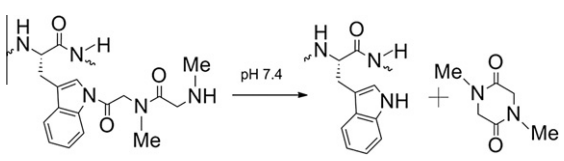
groups will improve the solubility. It would therefore, in many cases, be desirable to have a protecting group containing additional amino groups that increase the solubility of the peptide followed by fast formation of the desired peptide at physiological pH.

A well-known reaction in the peptide chemistry is diketopiperazine formation. The most studied examples being of dipeptide esters that can undergo a cyclization–elimination reaction resulting in a cyclic diketopiperazine dipeptide and the alcohol.[8] This reaction can occur as a side reaction during solid-phase peptide synthesis at the dipeptide stage, but it has also been used to design the cyclization-activated prodrugs for vinca alkaloids and purine and pyrimidine analogues.[9–11] Another interesting study showed that derivatization of the sparsely soluble immunosuppressive peptide cyclosporine A with different dipeptides resulted in cyclosporine A analogues with greatly improved solubility, and that cyclosporine A could be generated when the peptide was exposed to physiological pH.[12] In all these examples the dipeptide moieties were attached to a hydroxyl group but other studies have shown that even amide bonds in peptides can be cleaved slowly by the same reaction mechanism.[13]

In a project related to the synthesis of antimicrobial peptides with tryptophan residues we encountered problems in purification of the peptides and they were also difficult to dissolve for biological testing. We, therefore, became interested in the possibility that the indole nitrogen in tryptophan residues could be protected during the synthesis of the peptide with N-protected dipeptides. We hypothesized that such protecting groups would enhance the solubility of the peptide both during purification by HPLC and in the preparation of the sample for biological testing, yet would generate the active peptide rapidly when exposed to neutral pH in biological experiments (Scheme 1). The dipeptide for the diketopiperazine reaction is highly dependent on the dipeptide sequence and the rate of the reaction can vary by several orders of magnitude.[12] In general the reaction is fast for dipeptides with an N-alkylated peptide bond, probably as a result of a more favoured formation of cis-peptide bonds.[14] Therefore, we selected sarcosine (*N*-methylglycine) as the C-terminal moiety in the dipeptide. Sarcosine was also chosen as the N-terminal residue as it has been showed that the Z-Gly-Pro-ONp dipeptides can cyclize to the *N*-acyl diketopiperazine.[15] *p*-Nitrophenol is several orders of magnitude better as a leaving group than an indole but we could not exclude that the N-acylated nitrogen in primary amino groups could act as a nucleophile during the synthesis of the peptide when they are exposed to piperidine. Moreover, the attachment of dipeptides with N-terminal primary amino groups to the indole nitrogen would also require more complex synthetic procedures. The synthesis of Fmoc-tryptophan protected with the Boc-Sar-Sar group, Fmoc-Trp(Boc-Sar-Sar)-OH (**5**), on the indole nitrogen is outlined in Scheme 2.

The most direct, but not the most general, route to introduce an N-protected dipeptide onto the indole nitrogen on tryptophan involves acylation of the indole nitrogen with the activated dipeptide. If a proton is abstracted from the indole nucleus the nitrogen will become nucleophilic and can attack the electrophilic C-terminal carbonyl group in N^{α}-protected dipeptides with a good leaving group. This requires a strong base and in previous studies, including our own synthesis of Fmoc-Trp(Nmbu)-OH, the naked fluoride ion generated from solid KF and crown ethers has been used sucessfully.[6,16–18] The synthesis of Fmoc-Trp(Boc-Sar-Sar)-OH is described in the Supplementary data but requires some additional comments.

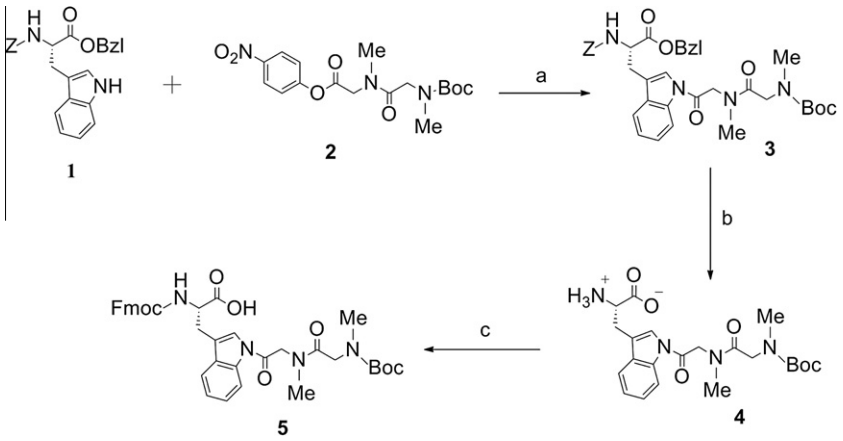


Scheme 1. Proposed result during the removal of the H-Sar-Sar group from the indole nitrogen of tryptophan at pH 7.4.

During the first step when Z-Trp-OBlz (**1**) is acylated by Boc-Sar-Sar-ONp (**2**) it is important to have high concentrations of the reactants. We found that when the reaction was performed with Z-Trp-OBzl (4 g) and dry THF (50 ml) was used as the solvent, only small amounts of the product were formed. Therefore, Z-Trp-OBzl, Boc-Sar-Sar-ONp and 18-crown-6 were dissolved in a very small amount of dry THF by gentle heating, *N*,*N*′-diisopropylethylamine (DIEA) was added, the solution was placed in an ice-bath followed by the addition of anhydrous KF. After 90 min at room temperature all the Boc-Sar-Sar-ONp had been consumed and peptide **3** could be readily purified by dry flash chromatography. After the catalytic hydrogenation of **3** we encountered problems separating **4** from the catalyst both by filtration and centrifugation. These problems could be eliminated if the use of a Teflon stirring bar was avoided during the reaction. The progress of the hydrogenation of **3** should also be carefully monitored by HPLC as a small amount of by-product starts to accumulate at the end of the reaction. This side-reaction could be minimized if the hydrogenation was interrupted just prior to completion. The small amounts of partly hydrogenated material could be removed by extraction of the solid material with diethyl ether.

During the synthesis of peptides with Fmoc-Trp(Boc-Sar-Sar)-OH there is a possibility that the Boc-Sar-Sar moiety could be cleaved by the nucleophile piperidine. To what extent this reaction will restrict the use of this protecting group is difficult to predict from the literature. It was shown by Arai and co-workers who used indole as protecting groups for carboxylic acids that a complete cleavage required 3 M NaOH in methanol for 3.5–35 h depending on the carboxylic acid used in the experiment.[19] On the other hand, it is well known that the N^{in}-formyl group is readily cleaved by nucleophiles. Previous results from this laboratory have shown that the Boc-4-(*N*-methylamino)butanoyl group was stable to 20% piperidine for up to 24 h.[6] In order to investigate the stability of the Boc-Sar-Sar group to piperidine, the model peptide Arg-Trp(Boc-Sar-Sar)-Ala-Arg-Tyr-Ala-OH attached to a 2-chlorotrityl resin was treated with 20% piperidine in NMP overnight followed by cleavage of the peptide from the resin with TFA (Fig. 1). This resulted in the partial cleavage of the Boc-Sar-Sar group, 14.4% of the Boc-Sar-Sar group was cleaved, as measured by absorption at 280 nm. This rate of cleavage by piperidine corresponds to the loss of 0.1% of the protecting group during a Fmoc group removal over 10 min (Fig. 1B). We, therefore, conclude that the Boc-Sar-Sar group is sufficiently stable to be used in the synthesis of most peptides using Fmoc chemistry.

In order to investigate the rate of cleavage of the N^{in}-Sar-Sar group at physiological pH the purified model peptide Arg-Trp(Sar-Sar)-Ala-Arg-Tyr-Ala-OH isolated as the TFA salt was exposed to HKR buffer at pH 7.4 followed by the acidification with TFA (Fig. 2). When the peptide was injected into the HPLC apparatus two peaks were observed corresponding to the original peptide with the N^{in}-Sar-Sar group and a second peptide with a 142 amu lower molecular mass corresponding to a peptide where the N^{in}-Sar-Sar group had been lost. The reaction was fast, after 2 min at room temperature 41% of the starting material had been converted into the fully deprotected peptide as measured by the absorption at 280 nm. Longer periods of exposure to physiological pH resulted in a decreased relative area of the protected peptide and after 20 min only one peak was observed in the HPLC elution profile, that is, the peptide in which the indole group had been deprotected. It is noteworthy that the deprotected peptide showed increased retention on the column indicating that the protected peptide was more polar and water soluble than the deprotected peptide (Fig. 2).



Scheme 2. The synthesis of Fmoc-Trp(Boc-Sar-Sar)-OH. (a): KF/18-crown-6/*N*,*N*′-diisopropylamine in THF, (b): H_2/10% Pd on charcoal in MeOH, (c): *N*,*N*′-diisopropylethylamine, chlorotrimethylsilane, Fmoc-Cl in CH_2Cl.

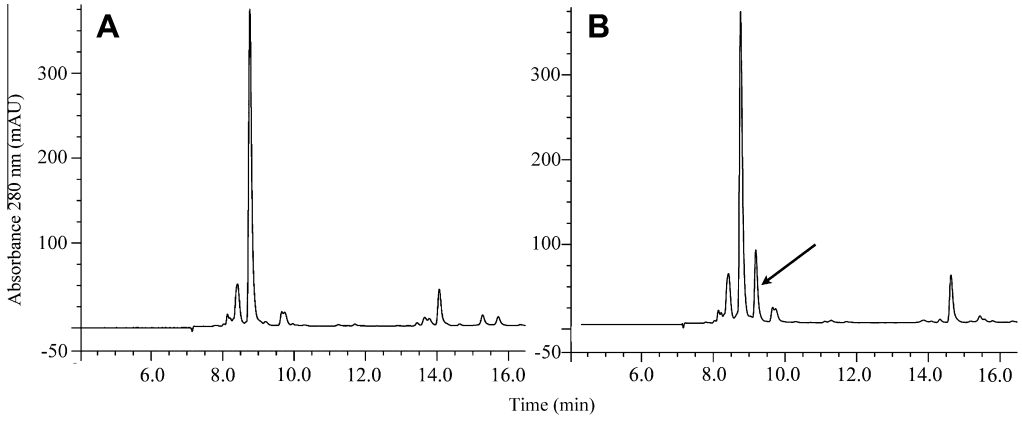


Figure 1. HPLC elution profile showing treatment of the peptide H-Arg-Trp(Sar-Sar)-Ala-Arg-Tyr-Ala-OH in 20% piperidine in NMP for 15 min (A), compared to treatment of the same peptide with 20% piperidine in NMP for 14 h and 40 min (B). The overnight treatment resulted in an additional peak (see arrow) corresponding to the deprotected product.

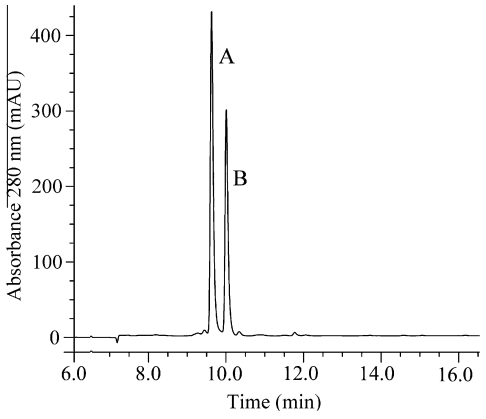


Figure 2. HPLC elution profile of the peptide H-Arg-Trp(Sar-Sar)-Ala-Arg-Tyr-Ala-OH exposed to pH 7.4 for 2 min at room temperature. The deprotected peptide H-Arg-Trp-Ala-Arg-Tyr-Ala-OH (B) shows an increased retention time indicating that the protected peptide (A) is more polar than the deprotected peptide (B).

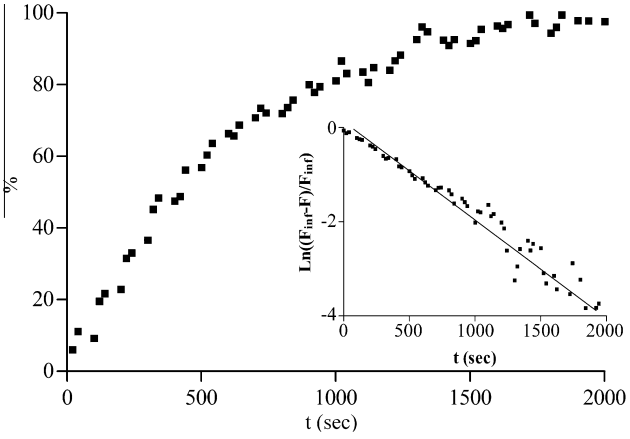


Figure 3. Cleavage of the H-Sar-Sar-group from the H-Arg-Trp(Sar-Sar)-Ala-Arg-Tyr-Ala-OH peptide as measured by the increase in fluorescence at 360 nm at pH 7.4. Inset: First order kinetics of the cleavage of the H-Sar-Sar group from the tryptophan residue.

A more accurate determination of the kinetics of the cleavage can be obtained by fluorescence spectroscopy (Fig. 3). Tryptophan emits fluorescence at 353 nm while the N^{in}-Sar-Sar group does not.[6] This will allow a direct measurement of the cleavage of the Sar-Sar protecting group without using HPLC separation. Using this method the first order rate constant for cleavage of the Sar-Sar protecting group from the H-Arg-Trp(Sar-Sar)-Ala-Arg-Tyr-Ala-OH peptide was determined as 2.1×10^{-3} s^{-1}, corresponding to a half-life of 5.5 min at pH 7.4 at room temperature.

We suggest that cleavage of the N^{in}-Sar-Sar group proceeds via an intramolecular cyclization reaction with the formation of deprotected tryptophan and *cyclo*[Sar-Sar] dipeptide. This mechanism is

supported by the observation that if Fmoc-Trp(Sar-Sar)-OH obtained after the cleavage of the Boc group with TFA is exposed to neutral pH, a product is observed in the HLPC elution profile that co-eluted with a commercial sample of *cyclo*[Sar-Sar] (Scheme 1).

In conclusion, the protecting group for tryptophan presented in this study offers no advantages as compared to the standard Boc protection used in Fmoc chemistry for the synthesis of the majority of tryptophan-containing peptides. However, for aggregation prone, hydrophobic peptides with poor solubility the use of Boc-Sar-Sar protected tryptophan can result in significant improvements both in the purification process and when the peptide is used in biological experiments. The Boc-Sar-Sar group might also find use in the design of pro-drugs of indole-containing compounds and possibly other structures containing heterocyclic nitrogen.

Acknowledgment

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Supplementary data

Supplementary data (experimental procedures for the synthesis of Fmoc-Trp(Sar-Sar)-OH, peptide synthesis and results from ^{13}C NMR and ^{1}NMR for Fmoc-Trp(Sar-Sar)-OH) associated with this Letter can be found, in the online version, at doi:10.1016/j.tetlet.2011.08.172.

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