Amino Acids
DOI 10.1007/s00726-016-2364-3

SHORT COMMUNICATION

# Efficient oxidation of *N*-protected tryptophan and tryptophanyl-dipeptides by in situ generated dimethyldioxirane provides hexahydropyrroloindoline-containing synthons suitable for peptide synthesis and subsequent tryptathionylation

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Received: 7 September 2016 / Accepted: 10 November 2016


**Abstract** A series of hydroxypyrroloindoline (Hpi) containing dipeptides along with the corresponding monomeric Hpi-α-amino acid (Hpi-2-carboxylate), were prepared by reacting a series of $N^{\alpha}$-protected-tryptophans in aqueous or biphasic [water/cyclopentyl methyl ether (CPME)] solutions containing Oxone® (potassium peroxymonosulfate) and acetone. This procedure avoids the tedious distillation of unstable dimethyldioxirane (DMDO), which is commonly used to oxidize indoles. Monomers $N^{\alpha}$-Boc-Hpi-OH and $N^{\alpha}$-Fmoc-Hpi-OH were readily incorporated by solid-phase peptide synthesis (SPPS) into a peptide containing a cysteine; in trifluoroacetic acid (TFA), the Hpi underwent intramolecular dehydrative condensation with the cysteine thiol to afford the anticipated tryptathionine crosslink. This eco- and user-friendly oxidative methodology greatly simplifies the synthesis of Hpi derivatives while enabling the synthesis of tryptathionine crosslinks characteristic of phalloidin and amanitin, two potent peptide toxins of present interest.

**Keywords** Dimethyldioxirane · Tryptophan oxidation · 3*a*-Hydroxypyrrolo[2,3-*b*]indoline · Peptide synthesis

Handling Editor: J. Bode.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00726-016-2364-3) contains supplementary material, which is available to authorized users.

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## Introduction

The 3*a*-hydroxypyrrolo[2,3-*b*]indoline (Hpi) (Fig. 1) motif arises from selective oxidation of tryptophan at position-3, represents a key toxicophore, and is found predominantly in the syn-*cis* configuration, in a large number of peptide and peptide-derived natural products (Ruiz-Sanchis et al. 2011; Roche et al. 2015; Lopez et al. 2008; Xu et al. 2014; Newhouse et al. 2010; Zhao et al. 2012; Ishikura et al. 2015; Deng et al. 2014, 2015; Zhu et al. 2015; Kamenecka and Danishefsky 1998a, b, 2001; Greenman et al. 2004; Buchel et al. 1998). The Hpi moiety can be constructed by treating tryptophan and its derivatives with several oxidants, most notably DMDO. Selective indole oxidation methods are thus of interest as they enable further elaboration to a host of tryptophan-derived natural products.

Hpi formation typically requires protection of the COOH to avoid oxindole formation. In some cases protection also affords highly diastereoselective oxidation that favors the syn-*cis* configuration, particularly in the case of a sterically bulky *tert*-butyl ester (Kamenecka and Danishefsky 2001; Savige and Fontana 1980). While protection with sterically bulky groups, e.g., a *tert*-butyl ester promotes high diastereoselectivity, multiple protecting group interconversions were required to incorporate Hpi into various natural products (Kamenecka and Danishefsky 2001). Such manipulations significantly reduce the overall yield in procuring a single diastereomer that can otherwise be obtained by chromatographic resolution from a non-diastereoselective oxidation. Indeed, to circumvent such interconversions and obviate the need for *C*-terminal protection, we explored the synthesis of Hpi-dipeptides (**2**) and (**3**) (May et al. 2005). These were procured by oxidizing $N^{\alpha}$-Tr-Trp-Xaa-OMe (**1**) dipeptides with freshly distilled DMDO in dichloromethane (Scheme 1) (Kamenecka

Published online: 19 November 2016

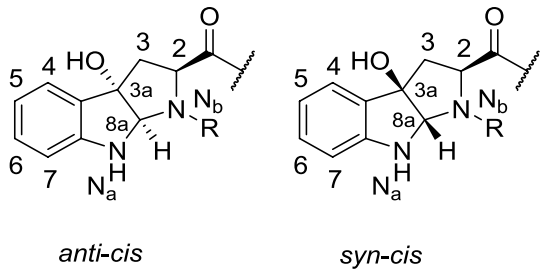
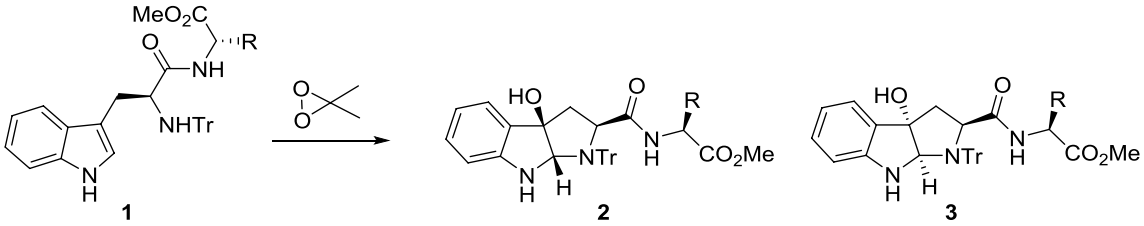


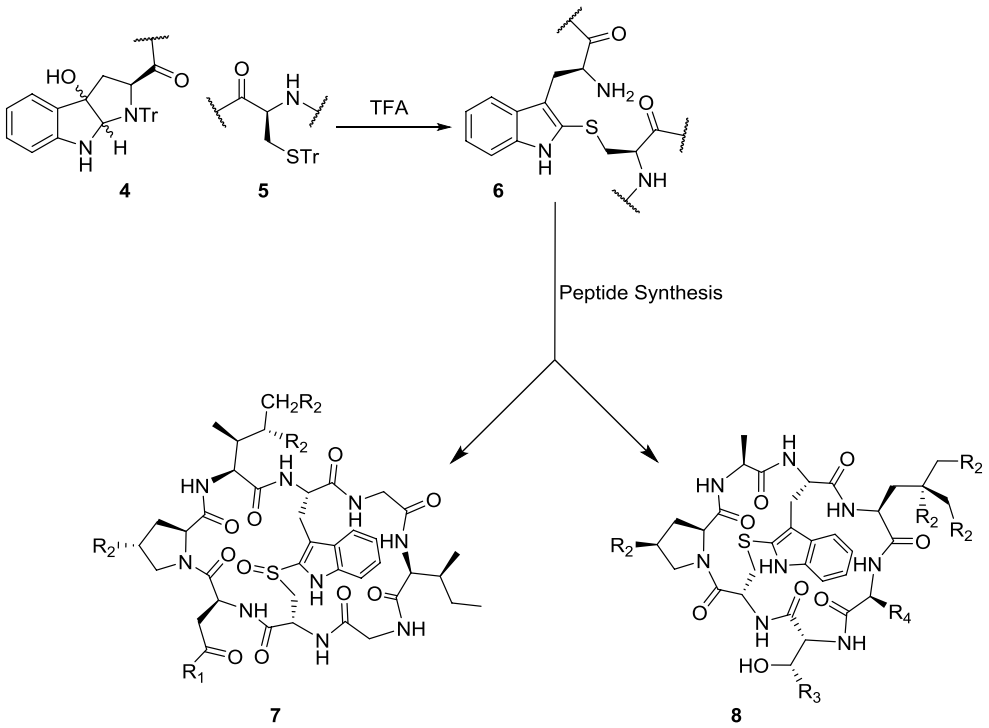

**Fig. 1** Structures of anti-*cis* and syn-*cis* diastereomers of 3*a*-hydroxypyrrolo[2,3-*b*]indoline (Hpi) (May et al. 2005)

and Danishefsky 2001; Mikula et al. 2013; Roche et al. 2015). Following saponification of the methyl ester, the *N*-protected Hpi dipeptides were compatible with standard peptide synthesis protocols.

Under acidic conditions, the Hpi (**4**) reacts with a cysteine thiol (**5**) to afford tryptathionine crosslink (indolyl-2-thiol ether) (**6**), that is found in amatoxin (**7**) and phallotoxin (**8**), two peptide toxins family of enduring interest (Scheme 2) (Wieland and Faulstich 1991; May and Perrin 2007; Anderl et al. 2012; Moldenhauer et al. 2012; Zhao et al. 2015). Because stereochemistry is abolished upon




**Scheme 1** *N*-1-Tr-3*a*-hydroxypyrrolo [2,3-*b*] indolyl amino acid methyl ester (Tr-Hpi-Xaa-OMe) syn-*cis* (**2**) and anti-*cis* (**3**) synthesis




**Scheme 2** Savige-Fontana reaction to afford a tryptathionine bridge (S-(2-tryptophanyl)cysteine) embedded in the synthetic preparation of amatoxin (**7**) and phallotoxin (**8**). Various derivatives are found in naturally occurring toxins with $R_1$=$NH_2$ or OH; $R_2$=OH or H; $R_3$=$CH_3$ or $CO_2H$; $R_4$=$CH_3$ or $CH(CH_3)_2$

tryptathionine formation, an unresolved mixture of syn-*cis* and anti-*cis* Hpi diastereomers may be used.

## Materials and methods

### General procedure A: synthesis of Hpi derivatives

A solution of 12 mM $N^{\alpha}$-Tr-Trp-Xaa-OMe dipeptide (1 equivalent) was prepared in cyclopentyl methyl ether. Then two volumes of Milli-Q water and one volume of HPLC grade acetone were added relative to 1 equivalent volume of CPME followed by a saturating amount of $NaHCO_3$ (140 equivalents) to keep pH at a value of approximately 8. To the vigorously stirred mixture maintained in an ice/water bath was added dropwise an aqueous solution of Oxone® (0.138 M) in three portions of 0.48 equivalents, 0.32 equivalents and 0.16 equivalents at 45 min intervals. Upon completion of reaction, the mixture was diluted with one volume of ethyl acetate (EtOAc). The organic layer was washed with saturated $NaHCO_3$ (aq) (3 × 0.5 volume) and brine (3 × 0.5 volume). The organic layer was dried over anhydrous $MgSO_4$, filtered out and evaporated to dryness under reduced pressure. The crude product was purified by flash chromatography on silica pretreated with triethylamine (TEA) using dry loading. A detailed procedure can be found in the supplementary material electronic file. Both syn-*cis* and anti-*cis* diastereomers were collected unless otherwise specified. All *N*-1-Tr-Hpi-Xaa-OMe compounds had been previously reported and were briefly characterized by $^1$H-NMR and HR-ESI–MS and found to be in accordance with our previous report.(May et al. 2005).

### General procedure B: synthesis of Hpi derivatives

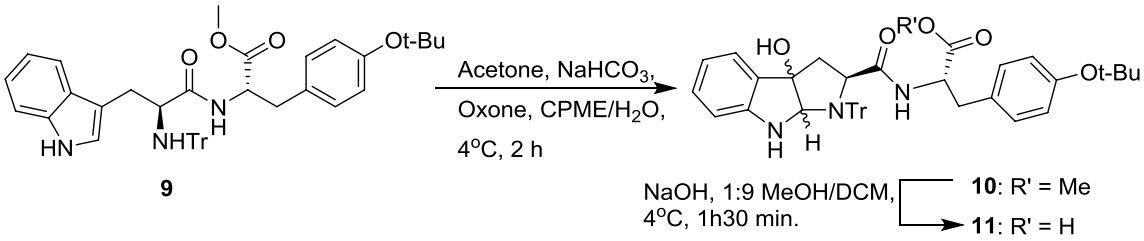
A 20 mM aqueous solution of $N^{\alpha}$-Boc-tryptophan (1 equivalent) or $N^{\alpha}$-Fmoc-tryptophan (1 equivalent) was dissolved in 22% (*v*/*v*) HPLC grade acetone in Milli-Q water and $NaHCO_3$ (20 equivalents) and retained in an ice/water bath. To the vigorously stirred mixture was added dropwise an aqueous solution of Oxone® (40 mM) in three portions of 0.36 equivalents, 0.36 equivalents and 0.30 equivalents at 45 min intervals in ice/water bath where pH 8 all along. Upon completion of reaction, the mixture was diluted with saturated aqueous $KH_2PO_4$ and 2% (*v*/*v*) aqueous $H_2PO_4$ until pH 3 was reached. The aqueous layer was extracted with EtOAc (7 × 0.15 volume) and the organic layers were pooled. The organic layer was washed with brine (3 × 0.17 volume), dried over anhydrous $MgSO_4$, filtered out and evaporated to dryness under reduced pressure. The crude reaction was subjected to flash chromatography on silica (acetic acid (AcOH)/dichloromethane (DCM)/EtOAc/Heptane or Pet Ether) using dry loading. A detailed procedure can be found in the supporting info. Both syn-*cis* and anti-*cis* diastereomers were simply collected as a mixture of diastereomers. Once purified by flash chromatography, acetic acid was not removable by standard procedure, and thus an additional work was done as follows. The dry mixture was dissolved in EtOAc, washed with saturated $KH_2PO_4$(aq) (0.33 volume) and the brine (0.33 volume). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness under reduced pressure to yield the pure carboxylic acid Hpi.

### General procedure C: saponification of methyl ester

A 0.09 M solution of *N*-1-Tr-Hpi-Xaa methyl ester (1 equivalent) in DCM was cooled in ice/water bath and a solution of 2.6 M NaOH (15 equivalents) in MeOH was added. The reaction was vigorously stirred and let warm up to RT for 1 h. The mixture was evaporated to dryness under reduced pressure. The product was purified by flash chromatography using dry loading on silica pretreated with TEA.

### General solid-phase peptide synthesis (SPPS) procedure

Manual solid-phase peptide synthesis was carried out in desalt Zeba™ spin column (Pierce) with vortex mixing on 2-chlorotrityl chloride (CTC) resin using standard Fmoc chemistry. Loading procedure: The vacuum-$P_2O_5$ dried resin (0.200 g) was resuspended in a solution of vacuum-$P_2O_5$ dried $N^{\alpha}$-Fmoc-Cys(Trt)-OH (4 equivalents, 0.15 M) in 1:1:3 (*v*/*v*/*v*) dry dimethylformamide (DMF)/dry acetonitrile (MeCN)/dry EtOAc and diisopropylethylamine (DIPEA) (9 equivalents). The mixture was shaken 5 h and 30 min, then MeOH (300 equivalents) was added and further shaken for 1 h. All soluble materials in solvent were recovered by filtration. The resin was washed with 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc (10 ml) and then resuspended in 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc (5 mL), shaken, drained by filtration and procedure was repeated six more times with fresh solvent. The filtration and shaking procedure was then repeated using 1:1 ethanol (EtOH)/EtOAc, dry MeCN, dry EtOAc and dry DCM. A loading test was performed to give on average a loading of 0.9 mmol/g. Fmoc deprotection procedure: If the previous step was not done in DMF then the resin was shaken 3 x 10 min in dry DMF and drained by filtration each time. The resin (0.200 g) was resuspended in 20% (*v*/*v*) piperidine in 0.5 M Oxyma in dry DMF (5 mL), shaken for 5 min, drained by filtration and procedure was repeated. The resin was washed with dry DMF (15 ml). The resin was resuspended in dry DMF (5 mL), shaken, drained by filtration and procedure was repeated six more times with fresh solvent. The filtration and shaking procedure




**Scheme 3** Typical Hpi preparation in biphasic solvent system

was repeated using 1:1 (*v*/*v*) EtOH/EtOAc, dry MeCN, dry EtOAc, dry DCM. A Kaiser test was performed. For peptide coupling, then the resin was further washed with 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc. $N^{\alpha}$-Fmoc-protected amino acid coupling procedure: the resin (0.200 g) was resuspended in solution made of $N^{\alpha}$-Fmoc-protected amino acid (4 equivalents, 0.15 M), Oxyma (4 equivalents), (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) (4 equivalents), DIPEA (12 equivalents) in 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc. The resin was shaken for a maximum of 1 h and a half. Upon completion of the reaction estimated by Kaiser test, the resin was drained by filtration. The resin was filtered with 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc (15 ml). The resin was resuspended in 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc (5 mL), shaken, drained by filtration and procedure was repeated six more times with fresh solvent. The filtration and shaking procedure was repeated using dry MeCN, dry EtOAc, dry DCM. A Kaiser test and loading test were performed. If the following step was $N^{\alpha}$-Fmoc deprotection, then the resin was further washed with dry DMF. Resin capping procedure: following each coupling step, a capping step was performed as follows. The resin (0.200 g) was resuspended in 1:2:2 (*v*/*v*/*v*) $Ac_2O$/TMP/dry EtOAc (5 mL) and shaken for 1 h, then drained by filtration. The resin was washed as done for Fmoc-protected amino acid coupling procedure. Hpi coupling procedure: the resin (0.200 g) was resuspended in solution made of Hpi derivative (2 equivalents, 0.07 M), Oxyma (2 equivalents), COMU (2 equivalents), DIPEA (6 equivalents) in 1:1:3 (*v*/*v*/*v*) dry DMF/dry MeCN/dry EtOAc. The resin was shaken overnight in dark and drained by filtration. The resin was washed as done for $N^{\alpha}$-Fmoc-protected amino acid coupling procedure.

### General Savige-Fontana tryptathionylation reaction procedure from solid-phase

The dry resin (0.200 g) was resuspended in TFA (5 mL) and shaken for 4 h. The supernatant was collected by filtration. The resin was drained by filtration with 1:1 (*v*/*v*) DCM/toluene (20 mL) and the filtrate was pooled with the previous filtrate. The resin was resuspended in 1:1 (*v*/*v*) DCM/toluene (5 mL), shaken and drained by filtration where the filtrate was pooled with the previous one. The shaking procedure was repeated six more times with fresh solvent. The shaking procedure was repeated ten times with each of the following solvents: DCM, MeCN, and EtOAc where all filtrates were pooled and evaporated to dryness under reduced pressure. The crude solid was dissolved in CPME and 10% (*v*/*v*) AcOH in water. The organic layer was extracted with 10% (*v*/*v*) AcOH in water and all aqueous layers were pooled. The resulting aqueous mixture was evaporated to dryness under reduced pressure and manual reverse phase column chromatography was performed on Sep-Pak (C18) gel (Waters, Delaware). The tryptathionine cyclic peptide was further purified by RP-HPLC (C18) with gradient of 20–100% solvent B over 30 min.

## Results and discussion

Production of DMDO typically requires careful distillation, but only recently has a method been developed to improve large scale procedure for DMDO preparation and storage (Mikula et al. 2013). However, in our hands, we have observed a need for periodic titration to assess a continuously waning concentration and rapid decrease in quality upon storage when used to oxidize tryptophan derivatives. Such considerations led us to investigate a simpler method for the preparation of Hpi-containing amino acids and dipeptides. Inspired by previous work in other laboratories, here we report the use of a simple admixture of Oxone in acetone in a biphasic reaction set-up which obviates the need for DMDO distillation (Scheme 3) (Hashimoto and Kanda 2002; Cheshev et al. 2006; Hussain et al. 2013; Zhu et al. 2015).

Initially, a cyclopentyl methyl ether (CPME) solution of 12 mM of $N^{\alpha}$-Tr-Trp-Xaa-OMe was diluted in an admixture of 1:1:2 (*v*/*v*/*v*) acetone/saturated aqueous sodium bicarbonate/water (pH 8.5). CPME was chosen based on its resistance to peroxide oxidation (Mouret et al. 2014). We found that a biphasic solution provided better results than the use of a homogeneous solution such as water/MeCN and

having a significant excess of acetone and bicarbonate was more critical than their actual concentrations. More important was the dropwise addition of aqueous Oxone solution in batches of 0.33 equivalents at 0–4 °C and pH >8. Following each addition, the reaction was deliberately stalled for 30–45 min; we found that addition of the Oxone all at once generated additional oxidized by-products, which were difficult to remove. Notably, without acetone, no Hpi is produced, suggesting that Oxone reacts with acetone to form DMDO in situ at a steady state rate. The reaction was retained at 0–4 °C to suppress oxindole formation along with other oxidized by-products, which proved difficult to separate from the desired Hpi products. In some cases, we deliberately added less than one equivalent of Oxone to avoid the formation of these difficult to separate oxidation products; while in such cases, the reaction did not go to completion; the desired Hpi compound could be cleanly separated from starting materials. Interestingly, tetrabutylammonium hydrogensulfate (TBAHS), added as a phase transfer catalyst, failed to improve either the yield or the apparent reaction rate (Lafont et al. 2011).

The reaction proved effective on a variety of dipeptide substrates, yielding an expected mixture of diastereoisomers that slightly favored the syn-*cis* configuration, and in overall yields comparable to those that we previously reported when using freshly distilled DMDO (Table 1; Supplementary information). Consistent with previous results, we observed highly selective oxidative cyclization of $N^{\alpha}$-Tr-Trp-Pro-OMe to the syn-*cis* diastereomer (**17a**) (Zhao et al. 2012). Overall, the oxidation method was sufficiently clean that the crude Hpi-dipeptide could be directly saponified to the corresponding carboxylate. For example, following basic work-up, the crude diastereomeric mixture *N*-1-Tr-Hpi-Tyr(*t*-Bu)-OMe (**10**) was saponified in hydroxide methanol (MeOH)/DCM solution to *N*-1-Tr-Hpi-Tyr(*t*-Bu)-OH (**11**) in 34% yield over two steps as a diastereomeric mixture (Theodorou et al. 2007). Using the same saponification method, but from a diastereomeric mixture (**10**) purified by flash chromatography, *N*-1-Tr-Hpi-Tyr(*t*-Bu)-OH (**11**) was prepared in 73% yield.

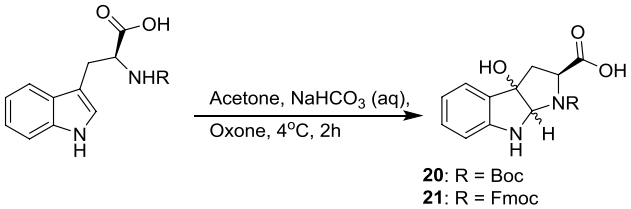
Gratified with the facility of this approach to indole oxidation within a dipeptide sequence, we extended this method to afford an Hpi synthon directly from monomeric tryptophan without protection of the COOH; such a monomer may find general use in peptide coupling, and in particular, for the synthesis of dipeptides such as Hpi-Trp and Hpi-Met, whose side chains are readily oxidized by DMDO (Savige and Fontana 1980). Thus, with bicarbonate buffered aqueous solution of $N^{\alpha}$-Boc-Trp-OH and acetone, a dropwise addition of Oxone gave *N*-1-Boc-Hpi-OH (**20**) in 32% yield as a diastereomeric mixture (Scheme 4).

Accordingly, *N*-1-Fmoc-Hpi-OH (**21**) and *N*-1-Fmoc-D-Hpi-OH (**21′**) were prepared in 46% and 33% yield from $N^{\alpha}$-Fmoc-Trp-OH and $N^{\alpha}$-Fmoc-D-Trp-OH, respectively. Although yields were low, this method allowed the oxidation of over a gram of $N^{\alpha}$-protected tryptophan with a free carboxylic acid group. Herein, the method led to the slight preference for the syn-*cis* diastereomer over the anti-*cis* in most $N^{\alpha}$-protected tryptophanyl-dipeptides and tryptophans, an observation that is consistent with our previous reports on the modest diastereoselectivity when using DMDO in dichloromethane.

**Table 1** *N*-1-Tr-Hpi-Xaa-OMe (**a**: syn-*cis*; **b**: anti-*cis*) dipeptide yield from in situ DMDO oxidation

| No. | Name | Yield (%) |
|---|---|---|
| **10a:b** | Tr-Hpi-Tyr(*t*-Bu)-OMe | 30:30 |
| **12a:b** | Tr-Hpi-Phe-OMe | 29:21 |
| **13a + b** | Tr-Hpi-Ala-OMe | 69[a] |
| **14a:b** | Tr-Hpi-Leu-OMe | 54:27 |
| **15a + b** | Tr-Hpi-Val-OMe | 83[a] |
| **16a + b** | Tr-Hpi-Ile-OMe | 68[a] |
| **17a** | Tr-Hpi-Pro-OMe | 29 |
| **18a + b** | Tr-Hpi-Gly-OMe | 56[a] |
| **19a:b** | Tr-Hpi-Thr(*t*-Bu)-OMe | 26:21 |

[a] Purified as a diastereomeric mixture

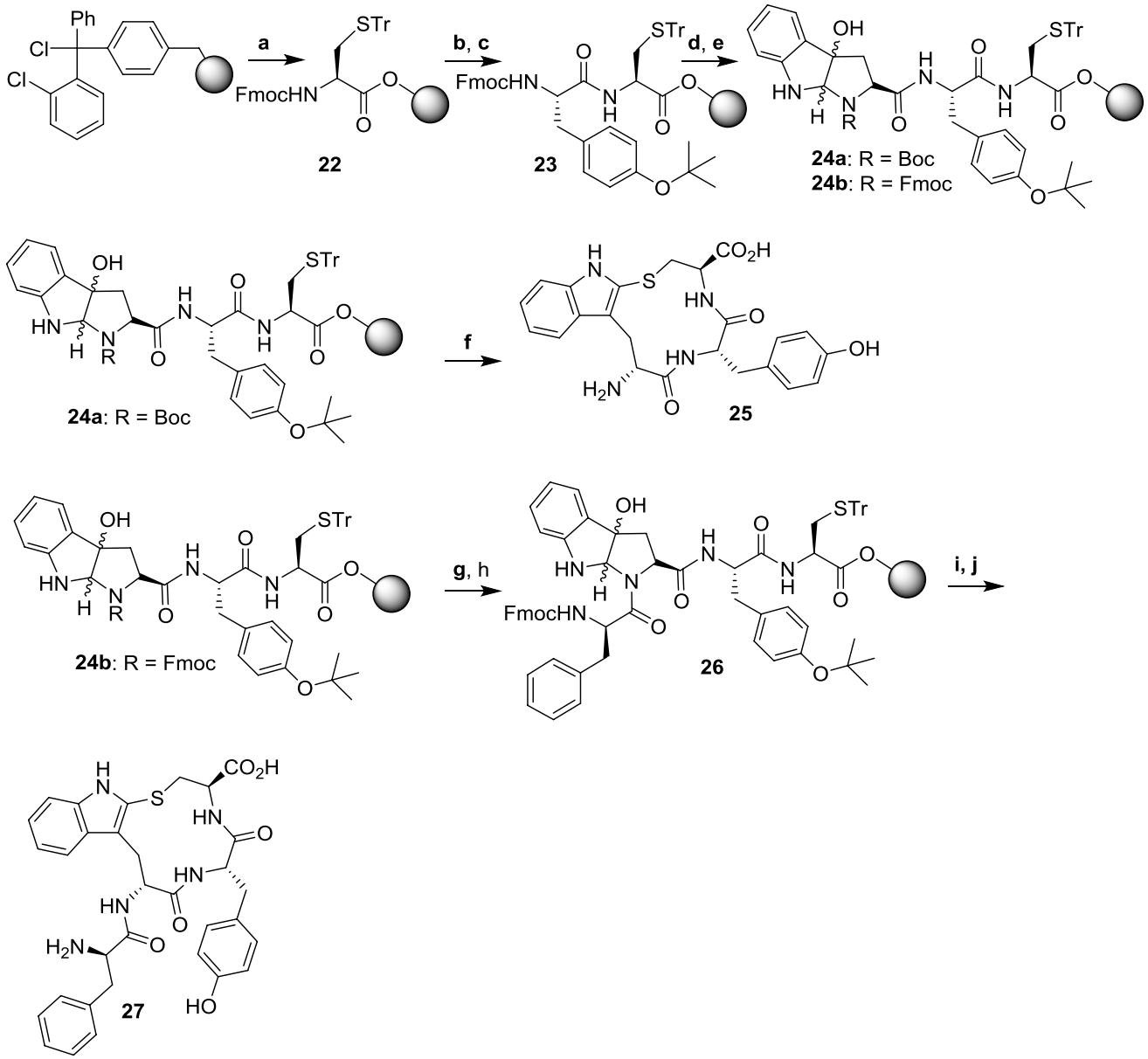



**20**: R = Boc
**21**: R = Fmoc

**Scheme 4** Synthesis of *N*-1-Boc-Hpi-OH (**20**) in 32% yield, *N*-1-Fmoc-Hpi-OH (**21**) in 46% yield and *N*-1-Fmoc-D-Hpi-OH (**21′**) in 33% yield

With an interest in forming tryptathionine crosslinks and to show by chemical correlation that this method afforded tryptathionine linked peptides, we prepared a diastereomeric mixture of *N*-1-Boc-Hpi-Tyr(*t*-Bu)-Cys(Tr) (**24a**) and *N*-1-Fmoc- Hpi-Tyr(*t*-Bu)-Cys(Tr) (**24b**) by standard SPPS on a chlorotrityl resin (Scheme 5).

Using COMU and Oxyma in a high proportion of ethyl acetate (60% in EtOAc), all peptide coupling reactions proceeded cleanly (MacMillan et al. 2013). Acetonitrile and DMF were added to improve solubility of the reagents. Oxyma was added to the piperidine solution during Fmoc deprotection to lower the risk of base-mediated side reactions.(Subiros-Funosas et al. 2012) An important application of *N*-1-Fmoc-Hpi was the ability to incorporate it into a peptide sequence by standard SPPS, followed by Fmoc deprotection with piperidine and then subsequent peptide




**Scheme 5** SPPS of tryptathionine peptide precursors and Savige-Fontana cyclization. Reagents and conditions: *a* $N^{\alpha}$-Fmoc-Cys(Tr)-OH, DIPEA, 1:1:3 MeCN/DMF/EtOAc, overnight; *b* 20% piperidine in 0.5 M oxyma in DMF, 1 × 5 min, 1 × 10 min; *c* $N^{\alpha}$-Fmoc-Tyr(*t*-Bu)-OH, COMU, Oxyma, DIPEA, 1:1:3 MeCN/DMF/EtOAc, 1 h 30 min; *d* repeated step *b*; *e* *N*-1-Boc-Hpi-OH (**20**) or *N*-1-Fmoc-Hpi-OH (**21**), COMU, Oxyma, DIPEA, 1:1:3 MeCN/DMF/EtOAc, overnight; *f* TFA, 4 h, after purification yield **25**; *g* repeated step *b*; *h* $N^{\alpha}$-Fmoc-D-Phe-OH, COMU, Oxyma, DIPEA, 1:1:3 MeCN/DMF/EtOAc, 1 h 30 min; *i* repeated step ***b***; *j* TFA, 4 h, after purification yield **27**

coupling as exemplified by tetrapeptide (**26**). Fmoc-D-Phe was chosen over other possible amino acids because the resulting tetrapeptide (26) was of interest in the development of additional peptide synthesis, currently ongoing in our laboratory. In all cases, tryptathionylation proceeded smoothly, with concomitant acid-mediated deprotection of side chains and the liberation of the peptide from the resin which corroborated previous results that an acylated Hpi can also undergo tryptathionylation. (May and Perrin 2008) Consequently, the procedure yielded H-(Trp-Tyr-Cys)c-OH (**25**) and H-D-Phe-(Trp-Tyr-Cys)c-OH (**27**) in good purity following Sep-Pak (C18) manual purification but were further purified by RP-HPLC (C18). The 16% yield of cyclic tripeptide (**25**) and 22% yield of cyclic tetrapeptide (**27**) were similar to previous reports on cyclic tryptathionine (May et al. 2005).

## Conclusion

In summary, herein we report a simplified method for Hpi preparation that uses cheap, commercially available, and environmental friendly reagents. Oxone by-products are easily neutralized into nontoxic sulfate, while DMDO is presumably produced in situ at a steady state rate. Salient points herein are that this method: (a) obviates the potentially hazardous, tedious, and often low yielding distillation of DMDO; (b) is amenable to scale up; (c) avoids COOH protection, albeit with loss of diastereoselectivity; and (d) in the case of water-soluble tryptophan derivatives, such as $N^{\alpha}$-Boc/Fmoc-Trp-OH, might be incorporated into a flow-based peptide synthesis system. As shown in scheme 5, the facile synthesis of monomeric N-1-Boc and N-1-Fmoc-Hpi-OH, provides a suitably protected monomeric Hpi that can be treated as a standard amino acid for coupling, which in turn allows access to Hpi-containing peptides that can otherwise contain oxidant-sensitive side chains (e.g., indoles, thioether). Evidence of this assertion is provided by the preparation of resin-bound peptide **23**, which contains a thioether, i.e., the tritylated cysteine. Hence, we suggest that this method merits consideration in procedures where DMDO or other oxidants, would be otherwise employed.

#### Complaince with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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