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Handling a tricycle: Orthogonal versus random oxidation of the tricyclic inhibitor cystine knotted peptide gurmarin

Rasmus Eliasen[a,b], Thomas L. Andresen[b], Kilian W. Conde-Frieboes[a,*]

[a] Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark
[b] Technical University of Denmark, Department of Micro- and Nanotechnology, Center for Nanomedicine and Theranostics, Produktionstorvet, Building 423, 2800 Kgs. Lyngby, Denmark

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ABSTRACT

Gurmarin is a 35 amino acid peptide with three disulfide bridges in an inhibitor cystine knot. It is found in the plant *Gymnema sylvestre*, and has been identified as a sweet taste inhibitor in rodents. In this article we provide an efficient route for the synthesis of gurmarin by a controlled random oxidation strategy. We compared two oxidation procedures to form the three disulfide bridges. In the first, based on random oxidation, reduced gurmarin was synthesized using trityl for cysteine protection, and oxidized for 48 h in a Tris–HCl buffer containing cystamine and reduced glutathione to facilitate disulfide scrambling. The second was based on step-wise deprotection followed by oxidation in which the cysteine pairs are orthogonally protected with *tert*-Butylthio, trityl and acetamidomethyl. To verify that the native gurmarin oxidation product was obtained, thermolysin cleavage was used. Cleavage of random oxidized gurmarin showed two possible disulfide combinations; the native and a non-native gurmarin disulfide isomer. The non-native isomer was therefore synthesized using the orthogonal deprotection-oxidation strategy and the native and the non-native gurmarin isomers were analyzed using UPLC. It was found that the random oxidation procedure leads to native gurmarin in high yield. Thus, the synthetic route was simple and significantly more efficient than previously reported syntheses of gurmarin and other cysteine rich peptides. Importantly, native gurmarin was obtained by random oxidation, which was confirmed by a synthetic approach for the first time.

1. Introduction

Gurmarin is a 35 amino acid peptide isolated from leaves of the plant *Gymnema sylvestre* [10]. The tertiary structure of gurmarin is determined by three disulfides in an inhibitor cystine knot (ICK) (Fig. 1) [7,8]. Gurmarin was identified as a sweet taste suppressor in rodents [10,16,17] and in Ayurvedic system of medicine the leaves and root of *G. sylvestre* has been used for treating diabetes and obesity [14]. This effect is believed to be caused by gymnemic acids, a mixture of at least 17 saponins, but gurmarin is also expected to be part of the effect [10,14]. Cystine knotted peptides have been suggested as scaffolds for drug development, due to their increased stability induced by the disulfide knot and their potential oral bioavailability [6,23].

The folding of cystine knotted peptides can essentially be done in two different ways. Using a redox buffer in which the disulfides scramble until they reach the thermodynamically most favorable conformation [4,17] or by using orthogonal cysteine side chain protection groups during solid phase peptide synthesis. The latter allow for selective formation of one disulfide at a time [3,12,19]. When using a redox buffer to oxidize a cystine knotted peptide, the peptide is dissolved in a slightly basic buffer containing a mixture of low molecular weight disulfides and sulfides, which facilitate scrambling of non-native disulfides combinations into the thermodynamically most favorable conformation [1,2,18]. The selective disulfide formation requires three pairs of orthogonal side chain protection groups. Cysteine protection groups can be labile to acid, base, metal ions or reducing agents [2,11]. The most commonly used cysteine protection group in Fmoc-SPPS is trityl [11], which can be removed with 95% TFA in the presence of scavengers [24]. Another common protection group is acetamidomethyl (Acm)

Abbreviations: 4-PDS, 4,4-dithiodipyridine; Acm, acetamidomethyl; DIPEA, diisopropylethylamine; DMSO, dimethylsulfoxide; EDT, ethanedithiol; ICK, inhibitor cystine knot; NMP, N-methylpyrrolidone; Pyr, pyroglutamic acid; *St*Bu, *tert*-Butylthio; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Trt, trityl; UPLC, Ultra Performance Liquid Chromatography.

* Corresponding author at: Protein and Peptide Chemistry, Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark. Tel.: +45 44434880; fax: +45 44444565.
E-mail address: kcf@novonordisk.com (K.W. Conde-Frieboes).

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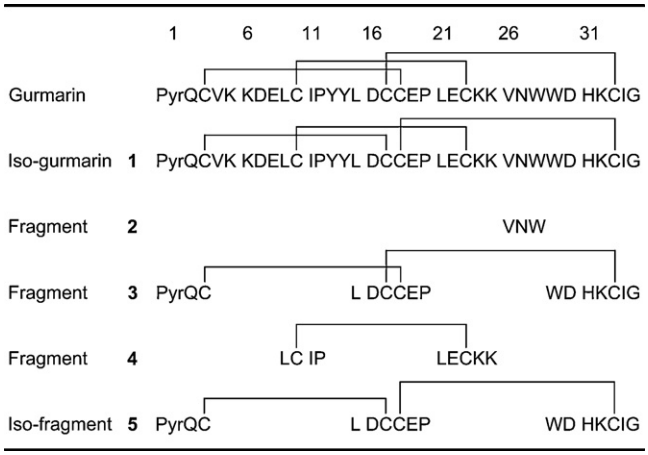

Fig. 1. The structures of and numbering of gurmarin, iso-gurmarin (**1**) and peptide fragments obtained in a thermolysin cleavage of random oxidized gurmarin.

[11], which is stable toward TFA. When treated with iodine, the Acm-protected cysteine is activated and forms a disulfide concurrently [20]. The two mentioned protection groups are stable toward reducing agents, and consequently *tert*-Butylthio (S*t*Bu) can be used in combination with these, since it can be removed with thiols or phosphines [11,22]. The free thiols can be oxidized using a variety of methods, both in solution and on-resin. It is important to maintain acidic pH during the oxidations to minimize disulfide scrambling. DMSO or 4,4-dithiodipyridine (4-PDS) can be used to oxidize thiols at acidic pH via activated disulfide intermediates [5,21]. By using 4-PDS it is possible to oxidize cysteines directly in the acidic acetonitrile/water solvent typically used in preparative HPLC purification.

The selective disulfide formation method ensures that the right disulfide combination is obtained. However, the procedure is often laborious and side reactions can occur during the continuous deprotections and oxidations [15]. The random oxidation method has the disadvantage that multiple disulfide combinations can be obtained. Nonetheless, it is expected that the native product will be formed in a random redox buffer oxidation, since the native product most likely has the highest thermodynamic stability [15]. However, this is not always the case [12]. Consequently, structure confirmation is essential when random oxidation is used. Because of the neighboring cysteines in ICK peptides, the structure confirmation cannot be done by an enzymatic fragmentation alone, since no cysteine specific proteases exist.

In this work gurmarin was synthesized both by random oxidation and by selective formation of the three disulfides using orthogonal cysteine side chain protection groups. The two different oxidation routes are illustrated in Fig. 2. To confirm the combination of the disulfides, the random oxidized gurmarin was cleaved with thermolysin to obtain one fragment (**4**) containing one disulfide from Cys10 to Cys23 and one fragment (**3**) containing two disulfides from Cys3 to Cys18 and Cys17 to Cys33. To ensure that fragment **3** in fact has the native conformation and not the non-native Cys3–Cys17 and Cys18–Cys33 conformation (**5**), a non-native gurmarin disulfide isomer (**1**) with this disulfide combination was synthesized using the orthogonal oxidation strategy. Finally, the two orthogonal oxidized peptides were compared with the random oxidized gurmarin by co-injection of the peptides on UPLC. Consequently, the disulfide pattern was confirmed by a synthetic approach.

2. Materials and methods

2.1. Materials

Resin and amino acids were purchased at Novabiochem, Germany. Thermolysin, glutathione, cystamine and 4-PDS were purchased at Sigma–Aldrich, Germany.

2.2. General solid phase peptide synthesis

The peptides were synthesized using Fmoc-chemistry using a preloaded Fmoc-Gly-Wang polystyrene LL resin in 0.1 mmol scale on a CEM Liberty microwave peptide synthesizer (CEM Corporation, NC) using standard protocols. Oxyma Pure, diisopropylcarbodiimide and amino acids were used for coupling in 5–10 fold excess over theoretical loading, and 5% piperidine in NMP with 0.05 M HOBt was used for Fmoc-deprotection. HOBt was added to reduce aspartimide formation of Asp16 [13]. Glu2 was coupled twice. Cys-residues and His31 were coupled twice at 50 °C.

2.3. Redox buffer oxidation

The peptide was synthesized as described in Section 2.2. Cysteines were protected with trityl. The peptide was cleaved with 92:4:2:2 TFA:ethanedithiol:water:triisopropylsilane, precipitated in diethyl ether (crude purity 54%) and purified on a Waters Delta Prep 4000 using a Waters XBridge Prep C18 OBD column (Waters Corporation, MA), 5 μm, 30 mm × 150 mm. Mobile phases were 0.1% TFA in water and 0.1% TFA in acetonitrile. Peptide concentration was determined by Chemiluminescent Nitrogen Detection using an Antek 8060 CLND HPLC detector (PAC, TX) [9]. Reduced gurmarin (**10**) was dissolved at 0.02 mM in a 0.1 M Tris–HCl (pH 7.8), 1 mM reduced glutathione, 1 mM cystamine redox buffer and stirred for 48 h. Gurmarin was purified as described above and fractions were analyzed on Waters Acquity UPLC using a BEH, 1.7 μm, 2.1 mm × 150 mm column and a gradient of 5–95% of acetonitrile containing 0.05% TFA against 0.05% TFA in water over 16 min, flow: 0.4 ml/min. Peptide MW was confirmed using Agilent 6230 TOF LC/MS (Agilent Technologies, CA). Peptide purity: 97.3%, retention time 5.8 min. Observed MW 1052.5 ($M+4H^+$)/4 (expected 1052.5).

2.4. Orthogonal oxidation

The peptide was synthesized as described in Section 2.2. Cys3 and Cys18 were Acm-protected, Cys10 and Cys23 were S*t*Bu-protected, while Cys17 and Cys33 were trityl-protected. S*t*Bu was removed (**7**) using 2–4 × 24 h 80:20:1 NMP:mercaptoethanol:diisopropylethylamine. The first disulfide was formed using 4:1 NMP:DMSO for 2 × 4 h. Peptide **7** was cleaved from the resin using the same conditions as described in Section 2.3, which also removed the trityl-protection groups. Peptide **8** was purified after cleavage from the resin. The second disulfide was formed using 10 equiv. of 4-PDS for 2 h and a peptide concentration of 0.05 mM directly in pooled fractions from the preparative HPLC diluted with water. After purification, the final disulfide was formed using 100 equiv. of I_2 in 4:1 acetic acid:water for 2 h and a peptide concentration of 0.05 mM (small amounts of acetonitrile and TFA from the preparative HPLC are included in the water part for simplicity. Exact amounts were not determined). The oxidation was stopped by adding ascorbic acid, the reaction mixture was diluted to 20% acetic acid and purified using a ODDMS 120A, 5 μm, YMC 4 mm × 125 mm column (FeF Chemicals, Denmark). All reactions were done at room temperature. The peptides were analyzed as described above. Synthesis of gurmarin. Purity: 76.6%, retention time 5.7 min. Observed MW 1052.5 ($M+4H^+$)/4 (expected