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Synthesis of Phakellistatin 13 and Oxidation to Phakellistatin 3 and Isophakellistatin 3

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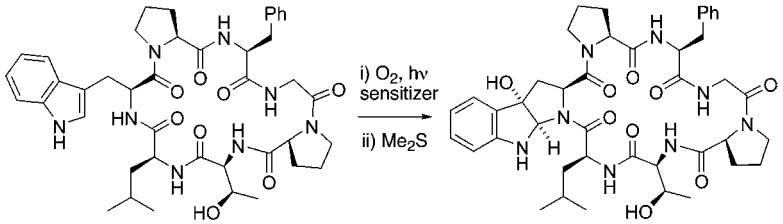
ABSTRACT



phakellistatin 13 **phakellistatin 3**

The natural product phakellistatin 13 *cyclo*-(TrpProPheGlyProThrLeu) was synthesized. Photosensitized oxidation of phakellistatin 13 gave the natural products phakellistatin 3 and isophakellistatin 3, demonstrating for the first time that a tryptophan residue can be directly converted to the corresponding 3a-hydroxypyrrolo[2,3-*b*]indoline in a full length peptide. Competitive oxidation of the indoline product was identified as the cause of low mass balance and is probably the source of low mass balance in the oxidative cyclization of all tryptamine derivatives.

Four groups of cyclic peptide natural products containing the 3a-hydroxypyrrolidino[2,3-*b*]indoline (Hpi) moiety have been isolated to date. These groups include himastatin,[1,2] phakellistatin 3 and isophakellistatin 3,[3] kapakahines C and D,[4] omphalotins B, C, and D,[5] and chloptosin.[6] Phakellistatin 3 was shown to inhibit P388 cell growth (ED_{50} 0.4 μM) while the diastereomer, isophakellistatin 3, was inactive. The Hpi residue presumably arises from oxidation of a Trp-containing peptide as opposed to enzymatic incorporation of the Hpi subunit. The recent isolation[7] of phakellistatin 13 (**1**) supports this hypothesis, wherein the Trp residue of phakellistatin 13 has been oxidized to Hpi in phakellistatin 3 (**2**) and isophakellistatin 3 (**3**, Figure 1).[4,5] However, there are no known synthetic transformations of Trp to Hpi in full length peptides.

Phakellistatin 13 exhibits potent, selective cytotoxicity. It is active against the hepatoma cell line BEL-7404 (ED_{50} < 12 nM) but inactive against the leukemia cell line HL-60. Since phakellistatin 13 is comprised of ribosomally compatible amino acids it may be mimicking an endogenous protein. The hydrophobicity of phakellistatin 13 (calculated *C* log *P* = 4.49) and phakellistatin 3 (calculated *C* log *P* = 3.77) suggest that the target could be intracellular.

The peptide sequence of phakellistatin 13 (*cyclo*-WPFGPTL) shares homology with several known human proteins. The selective cytotoxicity of phakellistatin 13 may be due to mimicry of one or more of these proteins, particularly proteins involved in protein recognition. We were further intrigued by the potential biogenetic relationship of the Hpi-containing product phakellistatin 3. Thus, we set out to study the oxidative cyclization of phakellistatin 13 to phakellistatin 3 and isophakellistatin 3 and elucidate the conformation of these molecules in solution.

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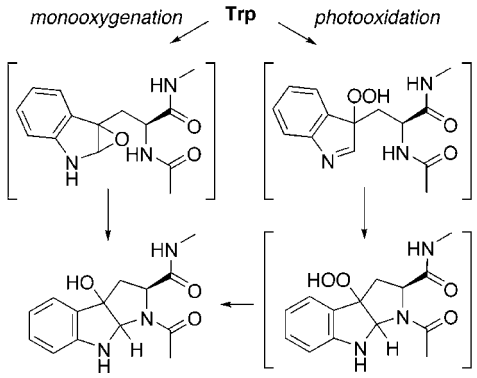
There are two principle synthetic methods for oxidative cyclization of tryptophan to Hpi: (1) reagent-based monooxygenation[8,9] and (2) photooxidative cyclization followed by peroxide reduction (Scheme 1).[10−12] Both methods have

Scheme 1



been widely applied to the amino acid tryptophan and simple derivatives of tryptophan but not to tryptophan residues in peptides. Multistep methods exist for the stereoselective synthesis of Hpi from suitably protected Trp derivatives, but incorporation into peptides is laborious.[9,13]

Conceptually, the most direct route to Hpi is oxidative cyclization of a Trp residue with an electrophilic oxygen atom source. Dimethyldioxirane (DMDO) is the most efficient oxygen atom donor for cyclization of suitably protected tryptophan derivatives, but even under ideal conditions it is unsatisfactory for oxidative cyclization of Trp in full length peptides.[9] The putative epoxide intermediates can rearrange to oxindolylalanine[14] and other products that are further susceptible to oxidation and oligomerization. Additionally, the desired product, an indoline, is likely to react with electrophilic oxidants faster than the indole starting material.[15,16] Not surprisingly, we found that oxidation of *N*α-acetyltryptophan methyl ester with one equiv. DMDO at −78 °C generates a complex mixture of products and starting material; reversed-phase HPLC reveals at least 10 new products. In contrast, oxidation of tryptamines can be efficient when the indole has a quaternary substituent at the 2-position that slows cyclization to the indoline.[17]

The highest yielding methods for Hpi formation involve a two-step sequence of photosensitized oxidative cyclization followed by peroxide reduction. For example, L-tryptophan has been oxidatively cyclized in water at low temperature using rose bengal to afford the Hpi derivative in 86% yield.[11] Photooxidations are capricious, and widely varying conditions have been reported for photooxidative cyclization of tryptophan derivatives. The highest isolated yield reported for the photooxidative cyclization of a tryptophan derivative, *N*α-Boc-tryptophan, is 90% (before drying).[12] Dipeptides (Xxx-Trp)[18] and diketopiperazines[19−22] fare much worse, giving Hpi derivatives in yields ranging from 11 to 39%. In our hands, photooxidative cyclization of *N*α-Boc-tryptophan on a small scale (100 mg) gave 50−60% yields of the Hpi derivative.[12] However, the photooxidative cyclization of *N*α-acetyltryptophan methyl ester gave none of the desired Hpi product on a 100 mg scale. However, by decreasing the temperature from 0 to −40 °C, the desired Hpi derivative was obtained in a 15% yield. We were unable to obtain better results on less than 1 mmol scale with any published conditions[11] or through independent variation of reaction parameters.

To investigate the oxidation of Trp to Hpi in the context of a full-length peptide, the synthesis of phakellistatin 13 was undertaken. Mindful of the fact that epimerization of activated amino esters can be problematic during slow couplings,[23] we chose to cyclize a linear peptide with glycine at the carboxy terminus. ProThr(O-*t*-Bu)LeuTrpProPheGly (**4**) was synthesized on chlorotrityl resin from Fmoc-protected amino acids using HBTU[24] and cleaved from resin using 1:1:98 TFA/EDT/CH_2Cl_2 (69%, after HPLC purification). The peptide was then subjected to macrocyclic ring closure at 1 mM in CH_3CN with HBTU and HOBt over 4 days to give *cyclo*-ProThr(O*t*-Bu)LeuTrpProPheGly (**5**) in 60% yield (Scheme 2). The substrate GlyProThr(O-*t*-Bu)LeuTrpProPhe (**6**), with an unhindered glycine at the *N*-terminus, cyclized in 16% yield without epimerization. Final deprotection of the Thr hydroxyl with 0.01:1:1 EDT/TFA/$CHCl_3$ proceeded in 94% yield to give synthetic phakellistatin 13 (**1**). The ^{1}H and ^{13}C NMR spectra of **1** were in agreement with those reported for the natural product except for a minor conformer present (4%) as indicated by the ^{1}H NMR, although no mention of multiple conformers was made in the original isolation paper. At 100 °C, the ^{1}H NMR revealed one set of signals. This work confirms that Trp1 has the natural L configuration, which could not be previously assigned

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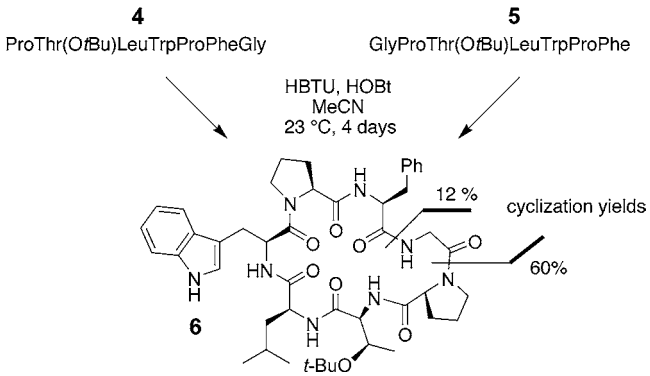
(24) Abbreviations used: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), and 1,2-ethanedithiol (EDT).

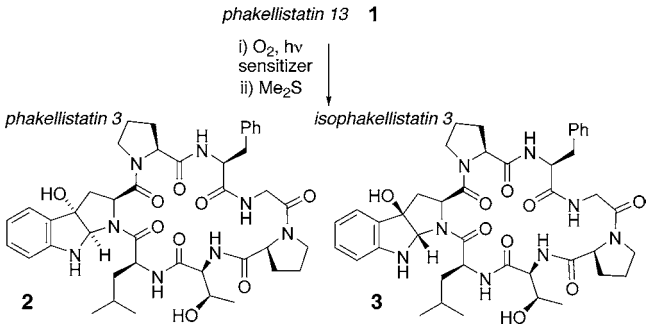
Scheme 2



because the acidic hydrolysis used for amino acid analysis destroyed the tryptophan residue.

Having prepared phakellistatin 13, we next turned our attention toward the oxidation of the Trp residue. Using the optimized photooxidation conditions, phakellistatin 13 was oxidized to a 1:1 mixture of phakellistatin 3 and isophakellistatin 3 in a combined yield of 20% (Scheme 3). Under

Scheme 3



the best HPLC conditions, phakellistatin 3 and isophakellistatin 3 elute as overlapping sharp and broad peaks, respectively. After multiple rounds of HPLC purification, phakellistatin 3 could be obtained in >95% purity. The ^{1}H and ^{13}C NMR of synthetic phakellistatin 3 in methanol-d_4 matched the published spectra for the natural product. In contrast, isophakellistatin 3 proved to be much more difficult to separate from phakellistatin 3. In fact, the NMR spectrum published for isophakellistatin 3 is actually contaminated with 20% of phakellistatin 3. After five rounds of HPLC purification, an 85:15 mixture of isophakellistatin 3 and phakellistatin 3 was obtained, giving a ^{1}H NMR spectrum that closely matched that previously published. When the photooxidation was applied to the Boc-protected indole **7** over 6 h, no reaction was observed.

In an attempt to explain the poor mass balance, two NMR experiments were conducted to test the sensitivity of the indoline intermediate to further oxidation under the conditions of the reaction (methanol-d_4, −40 °C). First, we treated

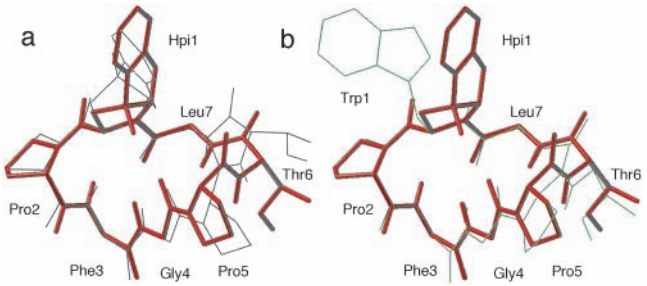


Figure 1. Superposition of (a) phakellistatin 3 (tube) with isophakellistatin 3 (wire) and (b) phakellistatin 3 (tube) with phakellistatin 13 (wire). Leu and Phe side chains were omitted for clarity.

indoline with *tert*-butyl hydroperoxide in the absence of light. No reaction was observed over 1 h. Next, we subjected indoline to our photooxidative conditions in methanol-d_4. Within 15 min, half of the indoline was consumed, giving primarily indole. This result clearly indicates that competitive photooxidation of the indoline products is a major reason for low mass balance in the photooxidative cyclization of tryptamine derivatives.[19−22] The problem with photooxidation is that it can generate two types of peroxide intermediates: a dioxetane that is poised to cleave to an *N*-formylkynurenine through a retro-[2 + 2] reaction or a 3-hydroperoxypyrrolidino[2,3-*b*]indoline[11] (Scheme 1) that is highly susceptible to further oxidation. To avoid this problem, one must use substrates that generate hindered/unreactive products or run the reaction to very low conversion.

The solution conformation of phakellistatin 13 was determined using a combination of ROESY and variable-temperature ^{1}H NMR. NOEs were observed from the Trp1-Hα to both the Phe3-NH and the Gly4-NH. The Gly4-NH appeared as a triplet (J = 3.7 Hz) suggesting a ϕ angle near 180°. Both of the Gly4 α-protons showed NOEs to the δ methylene protons of the adjacent Pro5, consistent with a *Z* amide conformation. In addition, the backbone NHs of Phe3, Gly4 and Leu7 exhibited low-temperature dependence ($\Delta\delta$ < 2 ppb/K) suggestive of intramolecular hydrogen bonding or solvent inaccessibility, whereas the backbone NHs of Trp1 and Thr6 were relatively large ($\Delta\delta$ > 4 ppb/K) suggesting exposure to solvent.

An attempt was made to obtain a diastereomerically pure sample of isophakellistatin 3 using the stereoselective cyclization reported for protected tryptophan derivatives using *N*-phenylselenophthalimide.[13,25,26] Unfortunately, after Boc-protection of the tryptophan side chain of peptide **6** an attempt to effect oxidative cyclization of the tryptophan residue with *N*-phenylselenophthalimide led to a mixture of over 10 products (Scheme 4).

Phakellistatin 3 exhibited one set of ^{1}H NMR signals in methanol-d_4 and two sets of signals in DMSO-d_6 (>95:<5).

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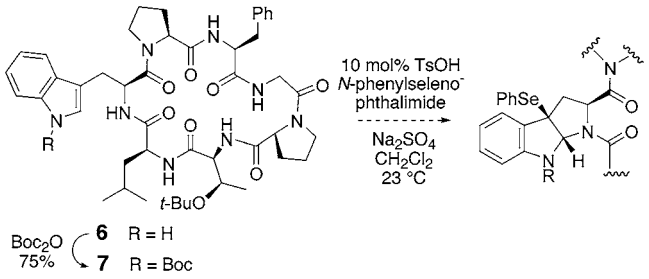
Scheme 4



Due to signal overlap, fewer resonances could be assigned. Two key NOEs could be discerned: between the Hpi β proton and one of the Pro2 δ protons; between the Thr_6 β proton and one of the Pro5 β protons. The Phe3 NH exhibited low temperature dependence ($\Delta\delta = 1.7$ ppb/K) whereas the Thr6 NH did not ($\Delta\delta = 4.1$ ppb/K). The Gly4 NH and Leu7 NH were not sufficiently resolved for VT analysis.

Amber*/H_2O does a good job of reproducing the conformation of isophakellistatin 3 for which there is a crystal structure. An unconstrained Monte Carlo search was used to generate plausible conformations for phakellistatin 3 and phakellistatin 13. The global minimum for phakellistatin 13 did not match all of the spectroscopic data; however, the family of conformers 0.7 kcal/mol above the global minimum (Figure 1) is consistent with all of the spectroscopic data. The global minimum identified for phakellistatin 3 was consistent with all of the spectroscopic data (Figure 1). Phakellistatin 3 and isophakellistatin 3 adopt similar backbone conformations except around Thr6 and Pro5; the threonine residue in isophekellistatin 3 is puckered toward the Hpi residue (Figure 1). The conformations of phakellistatin 3 and phakellistatin 13 are much more similar, with only subtle variations around Thr6 and Pro5.

Isophakellistatin is reported to be inactive against p388 cell lines whereas phakellistatin 3 and phakellistatin 13 are active against p388 and BEL-7404 cells, respectively. Since Pro2, Phe3, and Gly4 have nearly identical conformations in all three compounds it is tempting to attribute the differences in biological activity to the Hpi and Thr residues. The backbone conformations of these molecules lack common elements of protein secondary structure such as turns, sheets, or helices. Of the human proteins with sequence homology to phakellistatin 3 and 13, only RhoGAP proteins have published crystallographic data. Since the sequence FGPNL in P50 RhoGAP adopts a helical conformation, phakellistatins 3 and 13 do not appear to mimic the Rho-bound conformation of RhoGAP proteins.

In conclusion, phakellistatin 13 was synthesized confirming the absolute stereochemistry of the Trp residue. Phakellistatin 13 was oxidatively cyclized to phakellistatin 3 and isophakellistatin 3, demonstrating that full-length Trp-containing peptides can be oxidized to the corresponding Hpi-containing peptide. Over-oxidation of the Hpi residue accounts for the low mass balance in the oxidative cyclization of tryptophan residues in peptides. It is unclear whether the biological formation of phakellistatin 3 suffers from similar side reactions or whether Nature has found a way to cleanly generate Hpi residues. Spectroscopic data in conjunction with molecular modeling revealed a similar backbone conformation for all three cyclic peptides despite their markedly different biological activity against tumor cell lines. Further studies are required to elucidate the mode of action of phakellistatin 3 and 13.

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Supporting Information Available: Experimental procedures and spectroscopic data for compounds **1**−**7** and details of computational methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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