Abstract: A convenient synthesis in optically pure form of a derivative of L-proline is described, called photoproline, containing a diazirine group at position-4 of the pyrrolidine ring, starting from L-4-hydroxyproline. The use of Fmoc-L-photoproline in the synthesis of a cyclic peptidomimetic antibiotic demonstrates that this photoprobe can be incorporated into synthetic peptides using solid-phase Fmoc chemistry. Photoproline may be of wide value in the preparation of diverse peptide-based photoaffinity probes.
Synthesis and Application of PhotoProline - A Photoactivatable Derivative of Proline

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The paper is dedicated to Professor Heinz Heimgartner

Abstract
A convenient synthesis in optically pure form of a derivative of L-proline is described, called PhotoProline, containing a diazirine group at position-4 of the pyrrolidine ring, starting from L-4-hydroxyproline. The use of Fmoc-L-PhotoProline in the synthesis of a cyclic peptidomimetic antibiotic demonstrates that this photoprobe can be incorporated into synthetic peptides using solid-phase Fmoc chemistry. Photoproline may be of wide value in the preparation of diverse peptide-based photoaffinity probes.

Keywords: photoaffinity probe, peptide, receptor, peptidomimetic antibiotic, photolabel

Introduction
Photoaffinity labelling is a powerful tool for identifying targets of biologically active small molecules.\textsuperscript{1} A key requisite is that a photolabile group can be incorporated into the small molecule, along with a probe to allow later detection of photo-crosslinked receptors, without appreciable loss of biological activity and specificity. One of the most versatile photolabile groups for this purpose is the diazirine, due to its small size, and efficient and irreversible formation of a reactive carbene upon excitation at 350 nm.\textsuperscript{2, 3} Amino acid-based photoaffinity probes, including the phenylalanine analogues p-benzoylphenylalanine, p-azidophenylalanine and 4-[[3-trifluoromethyl]-3H-diazirin-3-yl]-phenylalanine have been known for some time,\textsuperscript{4,6} and more recently the alkylidiazirine amino acid analogs Photo-Leucine, and Photo-Methionine have been described.\textsuperscript{7, 8} Here we describe the synthesis and application of a related alkylidiazirine derivative of proline (PhotoProline), containing a diazirine group at position-4 of the pyrrolidine ring. A photolabile derivative of proline may be of special interest, since proline can have an important influence on peptide backbone conformation, and it frequently occurs within the energetically important epitopes in many biologically active peptides and proteins.
Results and Discussion
The synthesis of Fmoc-protected L-photoproline (PhotoPro), starting from relatively inexpensive L-trans-4-hydroxyproline, is shown in Scheme-1. The method used to install the diazirine group finds precedence in earlier published routes to PhotoLeucine and PhotoMethionine. The Fmoc-derivative was produced to allow incorporation of PhotoPro into synthetic peptides using standard solid-phase Fmoc chemistry. The enantiomer (Fmoc-D-PhotoPro) can be made in the same way, after epimerization of L-trans-4-hydroxyproline to D-cis-4-hydroxyproline, which is convenient to carry out on a mol scale following a published procedure.

Scheme-1. Synthesis of Fmoc-L- and D-PhotoProline.

We are interested in the application of PhotoPro to study the mechanism of action of a new family of peptidomimetic antibiotics active against the important human gram-negative pathogen Pseudomonas aeruginosa. The lead compound (L27-11, Figure-1) shows a minimal inhibitory concentration (MIC) against P. aeruginosa, including many multi-drug resistant clinical isolates, in the low nanomolar range.

Figure-1. Peptidomimetic antibiotic L27-11. The D-Pro-L-Pro template is highlighted.
The cellular target of this peptidomimetic antibiotic was shown recently to be the β-barrel outer membrane protein LptD, which is known to play a key role in outer membrane (OM) biogenesis by functioning with the lipoprotein LptE to transport lipopolysaccharide from the periplasm to the outer leaflet of the OM. Peptidomimetic L27-11 contains a D-Pro-L-Pro template, which is designed to stabilize β-hairpin-like conformations in the macrocycle. We, therefore, set out to replace the L-Pro residue in L27-11 by L-PhotoPro, in order to first investigate the effect of this change upon antimicrobial activity.

The synthetic approach to the L-PhotoPro analogue of L27-11 (Photo-L27-11) is shown in Scheme-2. The peptide synthesis was planned such that the PhotoPro could be incorporated close to the end of the solid-phase assembly process. However, we have subsequently used this amino acid for the synthesis of many other peptides, and so far no problems (e.g. instability) have been encountered with its use in Fmoc-solid-phase peptide synthesis. After assembly on the resin, the side-chain protected linear peptide chain was cleaved from the resin with mild acid, cyclized in dilute DMF solution, and the cyclic product was then deprotected with TFA. The final product was purified by reverse phase HPLC.


The antimicrobial activity of Photo-L27-11 against Pseudomonas aeruginosa ATCC 27853 was assayed using a standardized broth microdilution method. The MIC measured in Müller-Hinton broth was 0.008 μg/ml, which is essentially identical to that found for L27-11. Thus, the presence of the diazirine group has no effect on the potent antimicrobial activity of L27-11. We have also recently reported another analogue of this cyclic peptide, containing not only PhotoPro, but also with Thr replaced by a biotin tag (N-γ-(N-biotinyl-3-(2-(2-(3-aminopropyl)-ethoxy)-ethoxy)-propyl)-L-glutamine (Glu(biotinyl-PEG, Novabiochem)).
This photoprobe was used successfully in a photolabelling experiment to show that the peptidomimetic antibiotic binds to the OM protein LptD in *P. aeruginosa*.

Photoproline is readily available using the synthetic method reported here, and so may be of wide value in the preparation of diverse peptide-based photoaffinity probes.

**Experimental Section**

**N-Boc-L-trans-4-Hydroxyproline**

L-trans-4-Hydroxyproline (15 g, 114.4 mmol) was dissolved in water (60 ml) and dioxane (30 ml). Boc₂O (23.75 g, 109 mmol) and NaHCO₃ (12.08 g, 144 mmol) were added at pH ~ 7-8. The reaction was stirred overnight at room temp. The solution was filtered and dioxane was removed in vacuo. Excess Boc₂O was extracted into ice-cooled diethyl ether. The aqueous phase was acidified with 1 N HCl to pH 3. The product was extracted with ethyl acetate (4 ×) and the organic layers were combined, dried over Na₂SO₄, and the solvent removed in vacuo. The product was dried at high vacuum. Yield: 22.9 g (87%). m.p. 122-125°C. [α]²⁰°C₅₈₀nm = -79.4° (c = 9.9 mg/ml, H₂O). IR: υ (cm⁻¹) = 1734 (s), 1662 (s). ¹H-NMR (500 MHz, CDCl₃): δ (ppm) = 4.50-4.41 (m, 2H); 3.64-3.48 (m, 2H); 2.36-2.14 (m, 2H); 1.48-1.43 (d, 9H). ¹³C-NMR (DMSO-d₆): δ (ppm) = 174.29 + 173.78 (cis and trans rotamers), 153.64 + 153.10, 78.66, 68.39 + 67.69, 57.57 + 57.33, 54.55 + 54.24, 38.60 + 37.86, 27.97 + 27.77.

**N-Boc-L-4-oxoproline**

N-Boc-L-4-hydroxyproline (5 g, 21.6 mmol) was dissolved in 400 ml acetone. Jones reagent (37 ml, 98.8 mmol) was added drop-wise with cooling over 10 min and the reaction mixture was stirred for 2 h. Methanol (8.1 ml) was added drop-wise and the reaction mixture was then filtered through celite and concentrated in vacuo. EtOAc (270 ml) was added and the solution was again filtered through celite. The filtrate was washed with brine (6 × 100 ml). The organic phase was dried over Na₂SO₄ and solvent removed in vacuo. The product crystallized from ethyl acetate. Yield: 2.58 g (52.1%). m.p. 159-163°C (lit. 160-162°C). [α]²⁰°C₅₈₀nm = +19.7 (c = 31.6 mg/ml, acetone). IR: υ (cm⁻¹) = 1768 (s), 1751 (s), 1654 (s). ¹H-NMR (500 MHz, CDCl₃): δ (ppm) = 4.76-4.73 (t, 1H); 3.9-3.7 (m, 2H); 3.18-3.08 (m, 1H); 2.59-2.56 (d, 1H); 1.46-1.44 (2 × s, 9H). ¹³C-NMR (acetone-d₆): δ (ppm) = 209.07 + 208.39; 173.74 + 173.49, 155.07 + 154.32, 80.84, 57.14 + 56.51, 53.39 + 52.99, 41.73 + 41.19, 28.44 + 28.35. ESI-MS: m/z 228.0 (M-H⁺).

**N-Boc-L-4-diazirinylproline**

N-Boc-L-4-oxoproline (3.08 g, 13.5 mmol) was charged into a three necked-flask (100 ml) and ammonia was slowly condensed into the flask. The solution was refluxed for 5 h with stirring. The solution was cooled with a dry-ice bath and a solution of hydroxylamine-O-sulfonic acid in anhydrous methanol (8 ml, 1.84 M, 14.7 mmol) was added. The mixture was refluxed for a further 1.5 h. Anhydrous methanol (18 ml) was added while the reaction was cooled with a dry-ice bath. The solution was stirred overnight to allow the ammonia to evaporate. The resulting slurry was filtered through a sintered glass-funnel and the filter cake was washed twice with methanol (50 ml). The combined methanol phases were treated with triethylamine (1.88 ml, 13.6 mmol) and concentrated to ~15 ml. Another equivalent of triethylamine (1.88 ml, 13.6 mmol)
was added, the solution was cooled with an ice bath and titrated with a solution of I₂ in MeOH (0.1 M) until the solution remained a brown colour. The solvent was removed in vacuo and the resulting slurry was dissolved in water (50 ml). The solution was acidified to pH 2 and the product was extracted with ethyl acetate (4 × 50 ml). The organic phase was washed once with brine and dried over Na₂SO₄. The solvent was removed in vacuo and product was purified by flash silica chromatography (95-5, CH₂Cl₂-MeOH). Yield: 0.99 g (31%). m.p. 104-107°C. [α]²⁰⁺C₅₈₉nm = +16.0° (c = 10.1 mg/ml, MeOH). IR: v (cm⁻¹) = 1742 (s), 1636 (s), 1579 (w)¹⁴. ¹H-NMR (600 MHz, CDCl₃): δ (ppm) = 4.66-4.54 (2 × d × d, J=9.8 Hz & J=2.2 Hz, 1H); 3.24-3.20 (m, 1H); 3.20-3.06 (2 × d, J=12.6 Hz, 1H); 2.41-2.32 (m, 1H); 1.72-1.62 (2 × d × d, J=15.2 Hz & J=2.2 Hz, 1H); 1.46-1.44 (2 × s, 9H). ¹³C-NMR (CDCl₃): δ (ppm) = 177.63 + 175.90; 154.66 + 153.28; 81.89 + 81.44; 57.73 + 57.48; 48.40 + 48.03; 33.42 + 32.19; 30.80 + 30.30; 28.24 + 28.14. HR-ESI-MS: m/z (M⁺H⁺) 240.0993 (mcalc. = 240.0990).

**N-Fmoc-L-4-diazirinylproline**

N-Boc-L-4-diazirinylproline (0.99 g, 4.11 mmol) was dissolved in dioxane (20 ml) and concentrated HCl (2 ml) was added dropwise. The reaction was stirred for 1.5 h and the solvent evaporated in vacuo. The sample was dissolved in water and lyophilized to remove excess acid. The residue was dissolved in aq. Na₂CO₃ (9% w/v, 17.5 ml) and 0.9 eq. Fmoc-O-N-hydroxysuccinimide (1.24 g) in DMF/dioxane was added. The reaction was vortexed for 10 min and then water (150 ml) was added and unreacted Fmoc-O-N-hydroxysuccinimide was extracted with diethyl ether and EtOAc. The aqueous phase was acidified to pH 2 and the product was extracted with EtOAc (5 × 50 ml). The organic phase was dried with Na₂SO₄ and the solvent evaporated. The product was purified by flash chromatography first with silica using 94-5-1 CH₂Cl₂-MeOH-AcOH, and then using 90-10-0.25, EtOAc-n-hexane-AcOH as eluant. A white powder was obtained after lyophilization. Yield: 672 mg (46%). m.p. 113-116°C. [α]²⁰⁺C₅₈₉nm = +15.3° (c = 10.3 mg/ml, MeOH). IR: v (cm⁻¹) = 1736 (s), 1710 (s), 1677 (s), 1580 (w). ¹H-NMR (500 MHz, CDCl₃): δ (ppm) = 7.76-7.72 (m, 2H); 7.55-7.53 (m, 2H); 7.41-7.29 (m, 4H); 4.73-4.70 (m, 1H); 4.56-4.41 (m, 2H); 4.26-4.18 (2 × t, J=6.8 Hz, 1H); 3.32-3.23 (d × d, J=21.3 & J=12.7 Hz, 1H); 3.14-3.12 (d, J=12.3 Hz, 1H); 2.43-2.32 (m, 1H); 1.72-1.55 (2 × d × d, J=15.0 Hz & J=2.4 Hz, 1H). ¹³C-NMR (CDCl₃): δ (ppm) = 176.30 + 175.53, 154.99 + 153.79, 143.57 + 143.47, 141.35 + 141.32, 127.90 + 127.88, 127.17, 124.96 + 124.91, 120.10 + 120.05, 68.21 + 67.68, 57.88 + 57.35, 48.36 + 48.16, 47.09 + 47.06, 33.59 + 32.31, 30.75 + 30.12. HR-ESI-MS: m/z (M⁺H⁺) 362.1149 (mcalc. = 362.1146).

**N-Fmoc-D-4-diazirinylproline**

The methods used to prepare this compound were identical to those described above for the L-isomer, starting from D-cis-4-hydroxyproline.⁹ The product shows m.p. 113-116°C. [α]²⁰⁺C₅₈₉nm = -15.6° (c = 25.4 mg/ml, MeOH). IR: v (cm⁻¹) = 1747 (s), 1710 (s), 1677 (s), 1580 (w). ¹H-NMR (500 MHz, CDCl₃): δ (ppm) = 7.78-7.69 (m, 2H); 7.56-7.51 (m, 2H); 7.43-7.26 (m, 4H); 4.76-4.39 (m, 3H); 4.28-4.15 (2 × t, J=6.4 Hz, 1H); 3.34-3.21 (d × d, J=23.1 Hz & J=12.6 Hz, 1H); 3.16-3.10 (d, J=12.6 Hz, 1H); 2.45-2.33 (m, 1H); 1.74-1.52 (2 × d × d, J=15.0 & J=2.3 Hz, 1H). ¹³C-NMR (500 MHz, CDCl₃): δ (ppm) = 176.71 + 176.03, 154.92 + 153.84, 143.59 + 143.48, 141.34 + 141.31, 127.89 + 127.87, 127.17, 124.98 + 124.93, 120.09 + 120.05, 68.19 + 67.73, 57.90 + 57.41, 48.36 + 48.16, 47.08 + 47.06, 33.59 + 32.40, 30.77 + 30.12. HR-ESI-MS: m/z (M⁺Na⁺) 386.1113 (mcalc. = 386.1111).
Acknowledgements
The authors thank the Swiss National Science Foundation and the EU 7th Framework program (project NABATIVI) for financial support, and Annelies Meier for performing antimicrobial assays.

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