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Solid-Phase Synthesis of the Cyclic Peptide Portion of Chlorofusin, an Inhibitor of p53-MDM2 Interactions

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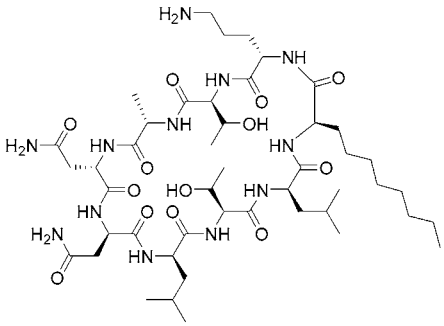
ABSTRACT



Chlorofusin Peptide

The first solid-phase synthesis of the chlorofusin peptide is described. The synthesis involved side-chain immobilization of N^{α}-Fmoc-Asp-ODmab. Synthesis of the linear peptide, initially incorporating racemic Ade8 and unsubstituted ornithine in place of the chromophore-bearing residue, was followed by cyclization on resin and peptide release to give a mixture of diastereomers. Resynthesis identified (by HPLC) the second isomer as analogous to the natural product. Initial biological assays, using an immunofluorescence method, suggest that the compounds are not cytotoxic but do not inhibit the p53/mdm2 interaction.

The tumor suppressor protein p53 is involved in the regulation of cell repair and proliferation and is mutated in over 50% of tumors.[1] In cancer cells that contain wild-type p53, over-expression of the regulatory protein MDM2 can suppress p53 activity.[2] MDM2 binds to the p53 DNA-binding domain and inhibits transactivation.[3] It contains a nuclear export signal and induces ubiquitination and subsequent degradation of the p53 protein.[4] Compounds that inhibit the binding of MDM2 to the tumor suppressor protein may restore p53-mediated cell cycle arrest or the apoptosis pathway and have potential as antitumor agents.[5] Chlorofusin (**1**, Figure 1) is derived from *Microdochium caespitosum* that has been shown to inhibit the p53/mdm2 interaction with an IC_{50} of 4.7 μM.[6] It consists of a cyclic peptide containing an L-ornithine side-chain that incorporates a densely functionalized chromophore.[6] Other key features of the structure include a D-2-aminodecanoic acid moiety, two D-leucines, two L-threonines, and both an L- and D-asparagine moiety.

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(1) Hainaut, P.; Hollstein, M. *Adv. Cancer Res.* **2000**, *77*, 81.
(2) Momand, J.; Zambetti, G. P.; Olson, D. C.; George, D.; Levine, A. J. *Cell* **1992**, *69*, 1237.
(3) Chen, J.; Marechal, V.; Levine, A. J. *Mol. Cell. Biol.* **1993**, *13*, 4107.
(4) Haupt, Y.; Maya, R.; Kazaz, A.; Oren, M. *Nature* **1997**, *387*, 296.
(5) Chène, P. *Nature Rev. Cancer* **2003**, *3*, 102.
(6) (a) Duncan, S. J.; Grüschow, S.; Williams, D. H.; McNicholas, C.; Purewal, R.; Hajek, M.; Gerlitz, M.; Martin, S.; Wrigley, S. K.; Moore, M. *J. Am. Chem. Soc.* **2001**, *123*, 554. (b) Duncan, S. J.; Grüschow, S.; Williams, D. H.; McNicholas, C.; Purewal, R.; Hajek, M.; Gerlitz, M.; Martin, S.; Wrigley, S. K.; Moore, M. *J. Am. Chem. Soc.* **2002**, *124*, 14503.

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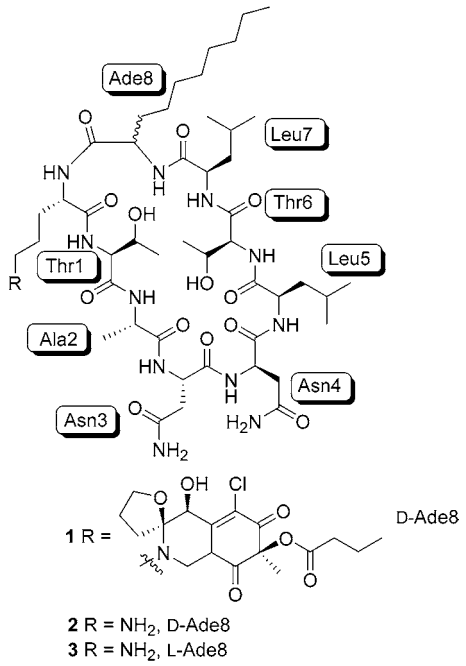
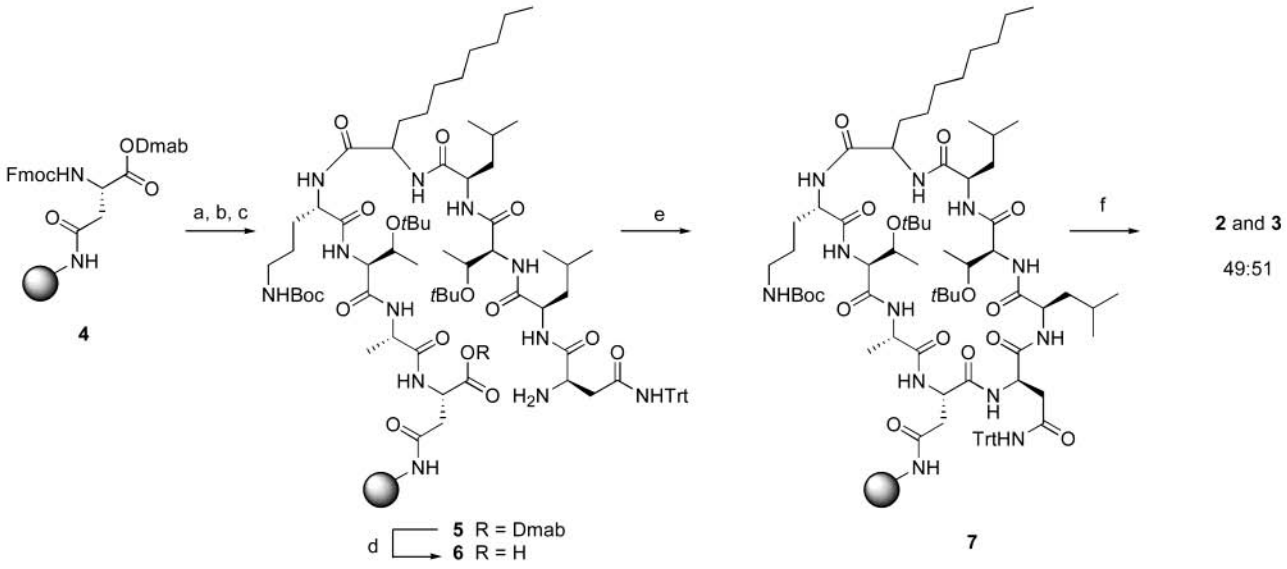


Figure 1. Chlorofusin (**1**) and the chlorofusin peptide **2**.

These latter two residues were not assigned in the structural analysis, although it was suggested that Asp3 was of the L-configuration.[6] To study the effects of chlorofusin and analogues as therapeutic agents, we have begun a study of its synthesis and a full biological characterization of the natural product, its substructures, and the structure−activity relationships required to develop a clinically useful agent. Herein, we describe the first solid-phase-based synthesis of the peptide portion of chlorofusin, in which the chromophore-bearing residue has been replaced by a simple ornithine (**2**, Figure 1). The synthesis involved "head-to-tail" cyclization[7] of the linear peptide immobilized through aspartic acid on a Rink amide resin. Subsequent release as the Asn3 residue was through standard TFA-induced methods. There have been few reports of this strategy for Asn-containing cyclic peptides.[8] The results presented here suggest the wide utility of the approach. We also report on an initial study of the biological effects of such peptides on p53 dynamics, as monitored by changes in the levels of transcriptionally active p53 in different phases of the cell cycle using multiparameter flow cytometry.

N^{α}-Fmoc-L-aspartic acid, protected at its α-carboxyl group as the Dmab ester,[9] was immobilized onto a Rink amide MBHA-derivatized polystyrene resin (substitution = 0.66 mmol/g) via its β-carboxylic acid group. The L-enantiomer was chosen, as it has been reported that Asn3 most likely has L absolute stereochemistry in chlorofusin on the basis of long-range NOE correlations.[6] Immobilization was achieved using in situ activation of the protected aspartic acid (2.5-fold excess over resin substitution) with an equimolar amount of HBTU and HOBt, in the presence of excess DIEA (5-fold over resin substitution). Following removal (20% v/v piperidine) of the N^{α}-Fmoc protecting group of resin-bound aspartic acid derivative **4**, the linear precursor **5** corresponding to the peptide sequence of chlorofusin was assembled using standard N^{α}-Fmoc-based solid-phase peptide synthesis (Scheme 1). The unsubstituted ornithine was incorporated as its N^{δ}-Boc-protected derivative. Both threonine residues

Scheme 1. Synthesis of **2**[a]



[a] Reagents and conditions: (a) (i) 20% v/v piperidine, DMF, rt, 2 × 10 min; (ii) Fmoc-Ala-OH, HBTU, HOBt, iPr$_2$NEt, DMF, rt, 2 × 20 min. (b) Repeat conditions (a) for Fmoc-Thr(tBu)-OH, Fmoc-Orn(Boc)-OH, Fmoc-DLAde-OH, Fmoc-DLeu-OH, Fmoc-Thr(tBu)-OH, and Fmoc-DLeu-OH and then Fmoc-DAsn(Trt)-OH. (c) 20% v/v piperidine, DMF, rt, 2 × 10 min. (d) 2% v/v N_2H_4·H_2O, DMF, rt, 3 × 3 min. (e) (i) DIC, HOAt, DMF, rt, 2 × 24 h; (ii) PyBOP, HOAt, iPr$_2$NEt, DMF, rt, 4 h. (f) TFA/iPr$_3$SiH/H_2O 95:2.5:2.5, rt, 2 h.

(Thr1 and Thr6) were incorporated as their *O-tert*-butyl-protected derivatives, and Asn4 (with D absolute stereochemistry) was incorporated as the protected trityl derivative. The unnatural 2-aminodecanoic acid (Ade8) residue was incorporated into the linear peptide precursor as a racemic mixture to allow synthesis of the L-Ade- and D-Ade-containing diastereomeric peptides in parallel, with subsequent resolution by reversed-phase HPLC during final purification.

On completion of synthesis of the linear precursor and final N^{α}-Fmoc deprotection (ca. 2−3 days), the α-carboxyl Dmab protecting group of Asn3 was removed using 2% v/v hydrazine hydrate in DMF.[9] Head-to-tail cyclization of resin-bound peptide **6** was accomplished using a 3-fold excess of DIC and HOAt[10] in DMF (2 × 24 h), monitoring completion with the Kaiser test.[11] In general, cyclization is more rapid with BOP/HOBt/*i*-Pr_2NEt but also leads to more extensive epimerization and oligomerization than the base-free conditions used.[12] However, to ensure that the ring closure had proceeded to completion, a precautionary third coupling step employing 3-fold excesses of PyBOP, HOAt, and *i*-Pr_2NEt was also carried out (Scheme 1).

After thorough washing and drying of the resin, the peptide was removed from the solid support (with simultaneous side-chain deprotection) by mild acidolysis with TFA. After freeze-drying, the crude cyclic peptide was obtained as a mixture of diastereomers (62 mg; 37% based on manufacturer's resin loading). The crude mixture was 59% pure as determined by analytical HPLC (L-Ade- and D-Ade-containing diastereomers as a 51:49 ratio).

Purification by semipreparative HPLC allowed efficient separation of the diastereomers, affording 12 mg (7%) and 9 mg (5%) of isomers **3** and **2**, respectively (HPLC retention times of 13.0 and 13.6 min, respectively).

The stereochemistry of the two diastereomers was ascertained by resynthesis using enantiomerically pure D-Fmoc-Ade-OH.[13] Both enantiomers were readily accessible through enzyme-mediated resolution of the N-chloroacetylated racemic mixture. Resynthesis of the single diastereoisomer **2** was exactly as that for the mixture. HPLC analysis of the product and coelution with the diastereomers separated in the first synthesis showed that the D-Ade compound exactly matched the compound of longer retention time.

The [^{1}H,^{1}H] NMR spectra of both peptides were assigned using standard procedures.[14] Both diastereomers tended to aggregate in solution, and experiments were carried out at 323 K for **2** and 308 K for **3**. The chemical shifts of the D-Ade8 peptide **2** were in good agreement with chemical shifts of chlorofusin (Table 1, Supporting Information).[6] Although the chromophore was not present, most of the NOE peaks characteristic for the natural product were detected in the NOESY spectrum of **2**, namely long-range cross-peaks between Leu5 and Thr1 and between Leu5 and Ade8, as well as the cross-peaks between Asn3 and Thr6. The presence of additional cross-peaks, for example, between Ala2 and Thr6 and between Thr1 and Thr6 suggested some differences from **1**. The aggregation of **2** under the conditions of the NMR experiment implied that the synthetic compound is a highly amphipathic molecule. A model based on NMR distance restraints derived from the high-intensity cross-peaks for the backbone and a few long-range cross-peaks (Figure 2a) predicted that the large hydrophobic chains of Ade8, Leu5, and Leu7 and the methyl groups of Thr1 and Thr6 would form a large hydrophobic surface on the top of the molecule.

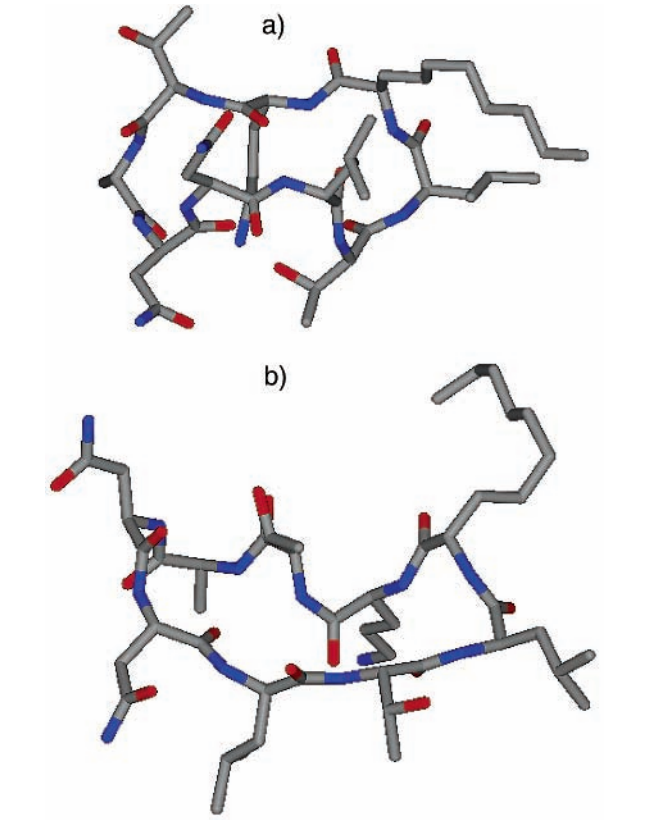


Figure 2. NMR-based lowest energy structures of (a) **2** (D-Ade) and (b) **3** (L-Ade).

The [^{1}H,^{1}H] NOESY spectrum of **3** had different features when compared with that for **2**. Particularly notable was the absence of long-range cross-peaks between Asn3 and Thr6 and between Ala2 and Orn9. The long-range peak across the cyclic backbone between Thr1 and Leu5 was still present.

(7) Blackburn, C.; Kates, S. A. In *Methods in Enzymology, Solid-phase Peptide Synthesis*; Fields, G. B., Ed.; Academic Press: Orlando, FL, 1997; Vol. 289, pp 175−198.
(8) Kates, S. A.; Sole, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 1549.
(9) Chan, W. C.; Bycroft, B. W.; Evans, D. J.; White, P. D. *J. Chem. Soc., Chem. Commun.* **1995**, 2209.
(10) Carpino, L. A.; El-Faham, A. *Tetrahedron* **1999**, *55*, 6813.
(11) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(12) (a) Plaué, S. *J. Pept. Res.* **1990**, *35*, 510. (b) McMurray, J. S. *Tetrahedron Lett.* **1991**, *32*, 7679. (c) McMurray, J. S.; Lewis, C. A.; Obeyesekere, N. U. *Pept. Res.* **1994**, *7*, 195.
(13) Koppitz, M.; Huenges, M.; Gratias, R.; Kessler, H. *Helv. Chim. Acta* **1997**, *80*, 1280.
(14) Wüthrich, K. *NMR of the Proteins and Nucleic Acids*; Wiley: New York, 1986.

A total of 92 unambiguous distance restraints derived from the NOESY spectrum employing 250 ms mixing time were used in a conformational search. The hydrophobic chain of L-Ade8 in the lowest energy conformation (Figure 2b) is above the cyclic peptide, but the backbone chain has a different bend. The polar groups of Thr1 and Thr6 are positioned between the hydrophobic chains of Leu5, Leu7, and Ade8, leading to a smaller amphipathicity of **3**, and consequently, the propensity for the aggregation is smaller compared with **2**.

Although the activity of MDM2/p53 inhibitors has traditionally been assessed by ELISA[6] or other in vitro assays,[15] a cell-based system is particularly attractive for the assessment of activity in live cells. U2OS, an osteosarcoma cell line, was selected for the live-cell peptide activity study since it contains wild-type p53 and overexpresses HDM2, the human homologue of MDM2. Importantly in U2OS cells, p53 is functional for transcriptional activation, showing the expected increased levels of p53 phosphorylation at Ser15 upon induction of genomic stress.[16] Changes in p53 dynamics were assessed in single cells by development of a sensitive immunofluorescence method. Attached U2OS cells were exposed to 50 μM peptide for 4 h, detached, and fixed in 4% paraformaldehyde for 30 min. Cell suspensions were processed for flow cytometry using rabbit anti-phospho-p53 serine 15-specific antibody (code 9284; Cell Signaling Technologies, USA). Binding was detected using a goat anti-rabbit IgG Alexa 488-conjugated antibody (Molecular Probes, Inc., OR) and cell cycle position detected using the DNA-specific far-red fluorescing dye DRAQ5 (Biostatus Ltd., UK). The results in Figure 3 show debris-gated contour plots for $>10^4$ cells. Analysis of the mean expression values for cells in early (G1), middle (S phase), and later (G2/M) cell cycle stages reveals no change in p53 dynamics resulting from peptide exposure. This result was confirmed by tracking the ability of treated cells to proliferate over a 48 h period using time-lapse imaging (data not shown).

In conclusion, we have described the first solid-phase-based synthesis of the chlorofusin peptide incorporating a free amino group in place of the natural product chromophore. The solid-phase synthesis is rapid and efficient, and immobilization of the peptide through the side-chain of aspartic acid allows on-resin cyclization. The two peptide diastereomers at Ade8 have different structures by NMR, although both have an amphipathic nature. Neither peptide activates p53 in the U2OS osteosarcoma cell line. Further structure−activity studies of the chlorofusin peptide are underway and will be disclosed in due course.

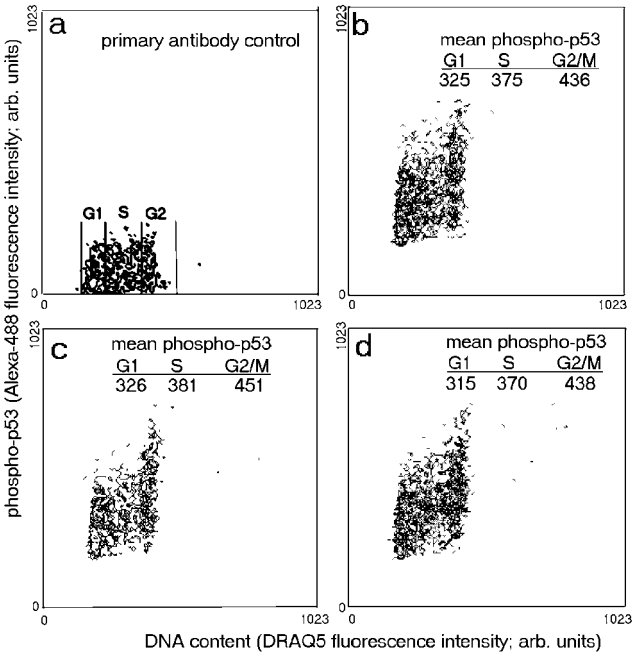


Figure 3. Flow cytometric tracking phosphorylation of p53 throughout the cell cycle in populations of U2OS osteosarcoma cells. (a) Phospho-specific antibody control; (b) untreated cells; (c) 50 μM **3** for 4 h; (d) 50 μM **2** (D-Ade8) for 4 h.

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Supporting Information Available: Protocol for the solid-phase synthesis and HPLC purification of **2** and **3**, as well as NMR and mass spectra and methods used for modeling of **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) (a) Surface plasmon resonance: Duncan, S. J.; Cooper, M. A.; Williams, D. H. *Chem. Commun.* **2003**, 316. (b) Polarization fluorescence assay: Knight, S. M. G.; Umezawa, N.; Lee, H.-S.; Gellman, S. H. *Anal. Biochem.* **2002**, *300*, 230.

(16) Campbell, K. J.; Chapman, N. R.; Perkins, N. D. *Biochem. Soc. Trans* **2001**, *29*, 688.