Synthesis of PAF, an antifungal protein from *Penicillium chrysogenum* by native chemical ligation

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Introduction

The *Penicillium chrysogenum* antifungal protein (PAF) is a cationic, cysteine-rich, 55-mer protein that inhibits the growth of a variety of filamentous fungi and has no toxic effect for mammalian cells [1, 2]. PAF is generally produced by *P. chrysogenum*, however, chemical synthesis of PAF is crucial for disclosing the disulfide pattern that stabilizes the fold and for the preparation of "chemical mutants".

Aims

Chemical synthesis, structural and biological investigations of the *Penicillium chrysogenum* antifungal protein (PAF) .

Synthetic strategies I.

1. Stepwise synthesis
2. Native chemical ligation

1. Stepwise synthesis

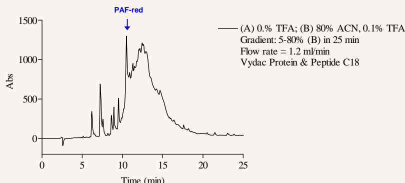
Solid-phase peptide synthesis was used.

Fmoc chemistry: failed.

Manual and microwave-assisted automated syntheses.
Resins: Wang and TentaGel resins.

Boc chemistry: low yield (~ 3% for the unfolded peptide)

Manual synthesis.
Resin: preloaded PAM resin.

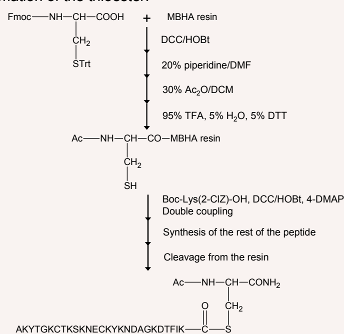


2. Native chemical ligation

Because of the low yield of stepwise synthesis, native chemical ligation [3] was the method of choice.

Synthesis of the thioester, PAF(1-27)

SPPS, Boc chemistry, MBHA resin
The SH group of Fmoc-Cys-OH was used as thiol for the formation of the thioester.



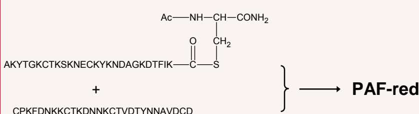
Cleavage conditions after the synthesis:
8% DMS, 2% anisole in liquid HF, -5-0°C, 45 min
The product was purified by semi-preparative HPLC.

Synthesis of PAF(28-55) CPKFDNKKCTKDNNKCTVDTYNNAVDCD

SPPS, Boc chemistry, preloaded PAM resin
Cleavage conditions after the synthesis:
8% DMS, 2% anisole, 2% p-cresol, 2% thioanisol
in liquid HF, -5-0°C, 45 min
The product was purified by semi-preparative HPLC.

Native chemical ligation

Conditions:
NH_4OAc buffer, pH 7.5; 6.7 mg/mL peptide concentration;
3% thiophenol; 4-5 hours



Synthetic strategies II.

1. Selective protection of cysteines
2. Non-selective protection of cysteines – oxidative folding

1. Selective protection of cysteines

Beside Mbzl, Acm and Fm protecting groups were used [4]. The synthesis was carried out according to that of PAF-red.
Ligation product: **PAF(Acm$_2$, Fm$_2$)-red**

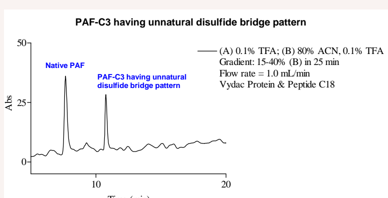
Formation of the 1st disulfide bridge:
NH_4OAc buffer, pH 7.5, 0.2 mg/mL peptide concentration, 24 h

Formation of the 2nd disulfide bridge – cleavage of Acm
80% MeOH/H_2O : 1M HCl (3:1) mixture
2.5 mg/mL peptide concentration, 5 equiv I_2, 30 min

Formation of the 3rd disulfide bridge – cleavage of Fm
80% piperidine/DMF; 1.5 mg/mL peptide concentration, 30 min

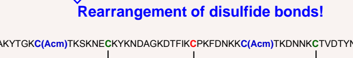
⇩ **Rearrangement of disulfide bonds!**

Unnatural disulfide bridge pattern!



Formation of the 2nd disulfide bridge – cleavage of Fm
80% piperidine/DMF; 1.5 mg/mL peptide concentration, 30 min

⇩ **Rearrangement of disulfide bonds!**



Unnatural disulfide bridge pattern!

2. Non-selective protection of cysteines – oxidative folding

Thiols of all of the 6 cysteines were protected by Mbzl.
Ligation product: **PAF-red**

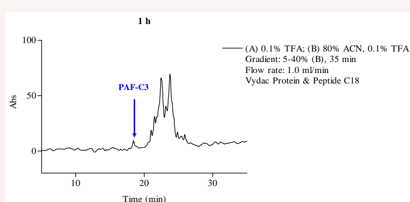
Unsuccessful oxidative folding methods:
Most of the methods have not provided the natural disulfide bridge pattern of PAF.

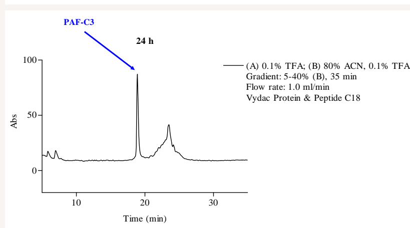
- I_2 / acetic acid solution
- I_2 / acetic acid solution, 6M guanidine
- Stirring with O_2 of air in acetate buffer (pH 7.5, 8.5 and 9.5)
- Stirring with O_2 of air in glycine buffer (pH 10.5)
- CLEAR-OX resin [5]

Successful oxidative folding:

Intensive stirring of PAF-red with O_2 of air in 0.1M NH_4OAc buffer (pH 7.5) in the presence of catalytic amount of cysteine [6]. Peptide concentration: 0.2 mg/mL.

Reaction mixture after 1 and 24 hours:



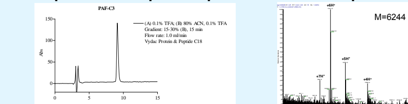


Sequence and supposed disulfide bond pattern of PAF

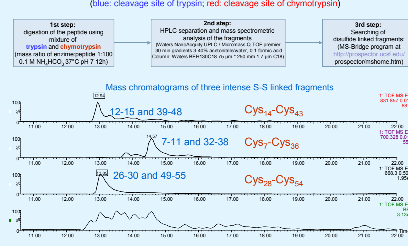
NMR investigations suggested two possible disulfide bond patterns for PAF: *abcabc* or *abbacc* [7]. Based on homology to other cysteine-rich antifungal proteins, the *abcabc* topology is more feasible.

Characterization of synthetic PAF

HPLC profile of the purified product **ESI-MS spectrum**



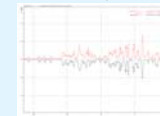
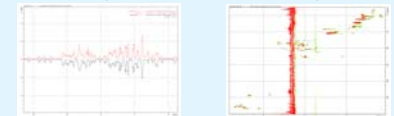
Strategy for identification of disulfide bridges in PAF



NMR investigations

NMR measurements prove that the structures of synthetic and native PAF are identical.

¹H NMR spectrum **¹³C-HSQC spectrum**

***In vitro* antifungal activity assay**

The minimal inhibitory concentrations (MICs) of native and synthetic PAF were 12.5 µg/ml, 6.25 µg/ml and 50 µg/ml to *Aspergillus nidulans* FGSC26, *Aspergillus niger* SZMC 601 and *Trichoderma longibrachiatum* UAMH 7955, respectively [8]. These data indicate that the *in vitro* antifungal activity of synthetic PAF on filamentous fungi is similar to which is exerted by native PAF.

Discussion

In this study we prepared PAF, a 55-mer antifungal protein stabilized by 3 disulfide bridges. Because of the very low yield of the stepwise synthesis, native chemical ligation was applied. The thioester of the N-terminal peptide fragment was prepared by following a new route: coupling of cysteine to a resin and using its sulfhydryl group as thiol function.

According to our experience, folding of PAF is under thermodynamic control. Thus, the native disulfide bridge pattern is not the most stable one. When selective protection was used for the cysteine thiols, basic treatment triggered rearrangement of the previously formed S-S bonds. When the thiols of all of the six cysteines were unprotected after the cleavage from the resin, most of the oxidative folding methods led to unnatural disulfide bridge pattern. The only method that produced native PAF was intensive stirring of oxygen of air to a solution of the reduced peptide in a pH 7.5 buffer in the presence of catalytic amount of cysteine.

MS, NMR spectra and antifungal activity assay proved that the chemically synthesized peptide is absolutely identical to the native one. Moreover, our synthetic results support the suggested disulfide bond pattern of PAF.

References

[1] Marx F, Haas H, Reindl M, Stoffler G, Lottspeich F, Redl B, *Gene*, **167,** 167-170, 1995
[2] Marx, F. *Appl Microbiol Biotechnol* **2004**, 65, 133-142.
[3] Dawson PE, Muir TW, Clarklewis I, Kent SBH, *Science*, **266**, 776-779, 1994
[4] Camarero JA, Giralt E, Andreu D, *Tetrahedron Letters*, **36**, 1137-1140, 1995
[5] Darlak K, Wiegandt Long D, Czzerwinski A et al., *J. Pept. Res.*, **63**, 303-312, 2004
[6] Szabó I, Schlosser G, Hudecz F, Mező G, *Peptide Science*, **88**, 20-28, 2006
[7] Batta G, Barna T, Gáspári Z, Sándor S et al., *FEBS Journal*, **276,** 2875-2890, 2009
[8] Galgóczy L, Kovács L, Vágvölgyi C, p 550-559. In Méndez-Vilas A (ed), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology Vol. 1, Microbiology Book Series-Number 2. Formatex, Bajadoz, Spain, 2010

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