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TOTAL SYNTHESIS OF THE LIPOPEPTIDE a-MATING FACTOR OF SACCHAROMYCES CEREVISIAE

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SUMMARY: The a-mating factor of Saccharomyces cerevisiae was synthesized using both solution phase and solid phase strategies. Structure of the final peptide was confirmed using amino acid analysis, fast atom bombardment mass spectroscopy and 400 MHz proton NMR. The synthetic farnesylated dodecapeptide, YIIKGVFWDPAC(S-farnesyl)OCH_3, exhibited chromatographic and spectroscopic properties identical to the natural pheromone and had significant biological activity at nanomolar concentrations.

INTRODUCTION: Thirty years ago Levi(1) recognized that chemical species in the culture supernatant of haploid yeast cells were involved in the mating process of Saccharomyces cerevisiae. In 1976 Stotzler et al. (2) determined the structure of the mating pheromone from the culture of α-haploids and this tridecapeptide, WHWLQLKPGQPMY, was synthesized shortly thereafter (3,4). In contrast the structure of the reciprocal factor from a-haploids remained elusive and it is only recently that Anderegg et al. (5) showed that the a-factor was a lipopeptide similar to pheromones from Tremella mesenterica (6) and Rhodosporidium toruloides (7). Interestingly, the two pheromones of S. cerevisae appear to be biosynthesized and secreted by distinct pathways (8,9). In order to confirm the structure of the a-factor and provide precursors for the study of its biosynthesis we undertook the synthesis of the lipopeptide

TyrIleIleLysGlyValPheTrpAspProAlaCys(S-farnesyl)OCH_3

which represents the mature a-factor encoded by the MFa1 gene (9). In this communication we report the total synthesis of the a-factor using both solution phase and solid phase strategies.

MATERIALS and METHODS : All protected amino acids were purchased from BaChem Inc, Torrance CA except for Boc-Asp(Ofm) which was synthesized

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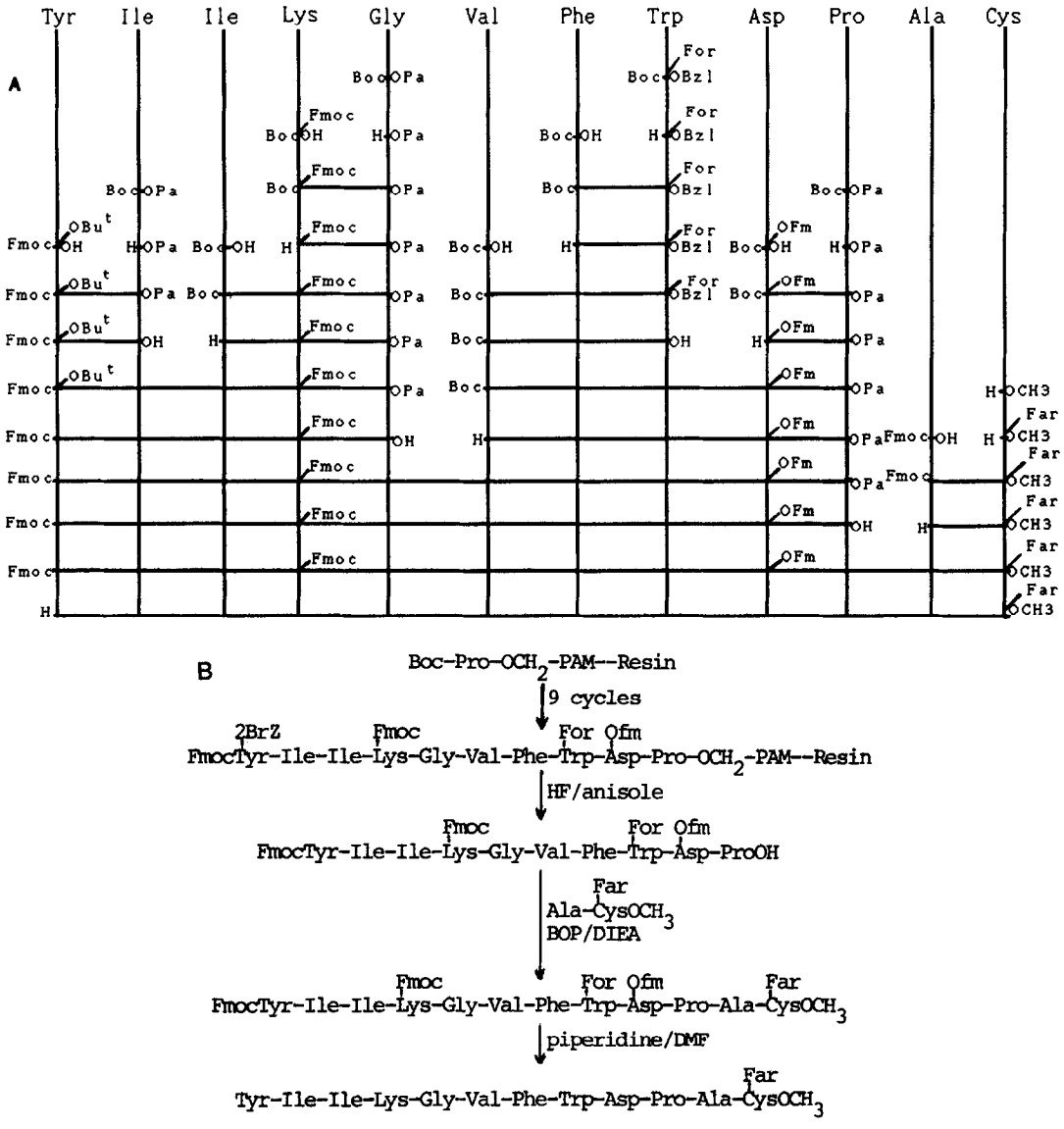
following a procedure provided by Dr. Arthur M. Felix , Hoffman LaRoche Inc. All reagents and solvents were the purest commercially available and were used as received. HPLC was run on a Waters Inc gradient system using C-18 columns. Detection was at both 220 nm and 254 nm.

Biological activity of the synthetic a-factor was measured in two ways using S. cerevisiae MATα cells. Various amounts of the mating factor were added to agar plates which were previously overlayed with S. cerevisiae RC757, a strain supersensitive to the pheromone. The plates were incubated at 30°C for 36 hours and observed for the presence of halos, indicative of peptide-induced growth arrest. The activity is reported as the lowest amount of peptide that causes inhibition of growth. The propensity of the pheromone to cause morphological changes in yeast cells was also determined (5) using wild type S. cerevisiae X2180-1B.

RESULTS

Synthesis of Peptides : The synthesis of the S. cerevisiae a-factor was carried out by both conventional solution phase synthesis (Scheme 1A) and a combination of solution phase synthesis and solid phase synthesis (Scheme 1B). The strategy underlying our synthetic approach was to incorporate the farnesyl moiety late in the synthesis and to avoid exposure of this moiety to acidic conditions. Furthermore, we chose not to add this moiety to a peptide containing both sulfhydryl and amino groups since this would have yielded a complex product mixture. Schemes 1A and 1B utilize various combinations of tert-butoxycarbonyl (Boc), 9-fluorenylmethoxycarbonyl (Fmoc), 9-fluorenylmethyl (Ofm), 2-bromobenzyloxycarbonyl (2-BrZ), formyl (For) and phenacyl (OPa) protecting groups. Cys(farnesyl)OCH_3 was made by reaction of L-cysteine methyl ester with farnesyl bromide, and then coupled with FmocAla to give Fmoc-Ala-Cys(S-farnesyl)OCH_3. The amine terminal decapeptide was synthesized either in solution or on a PAM resin (phenylacetamidomethyl resin). The solution phase synthesis of the decapeptide required both 3+2 and 5+5 fragment coupling. In the steps where 3+2 coupling was employed hydroxybenzotriazole (HOBt) was present to minimize racemization and side reactions. The 5+5 fragment coupling was achieved using BOP [benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate] as the coupling agent.

The protected dodecapeptide was assembled by 10+2 fragment condensation. The reaction was followed by HPLC and was complete within one hour using BOP as the coupling agent(10). By way of comparison very little coupling was observed when dicyclohexylcarbodiimide/HOBt was used as the coupling agent in a parallel reaction. After incorporation of Ala-Cys(S-farnesyl)OCH_3 the protecting groups were removed by treatment with piperidine in dimethylformamide and the a-mating pheromone was purified by reversed phase high performance liquid chromatography. The yield from the solid phase route was 25 percent whereas the conventional solution phase route gave a 28 percent yield for the 10+2 fragment coupling and subsequent



Scheme 1. Synthetic strategy for synthesis of a-factor. Panel A - solution-phase synthesis. Panel B - combination of solution-phase and solid-phase synthesis.

deprotection and purification steps. Products from both routes had identical chromatographic properties and 400 MHz ^{1}H-NMR spectra. The identical mobility of the products from both routes suggests that significant racemization did not occur during the solution phase synthesis of the decapeptide.

Characterization of Synthetic a-Factor: The solution phase and solid phase derived pheromones exhibited identical mobilities on reversed-phase HPLC (Fig 1) and eluted at 59-61 percent acetonitrile which is almost identical to the result reported by Betz et al. (11). The a-factor was also homogeneous on silica thin layers using both butanol:acetic acid:water

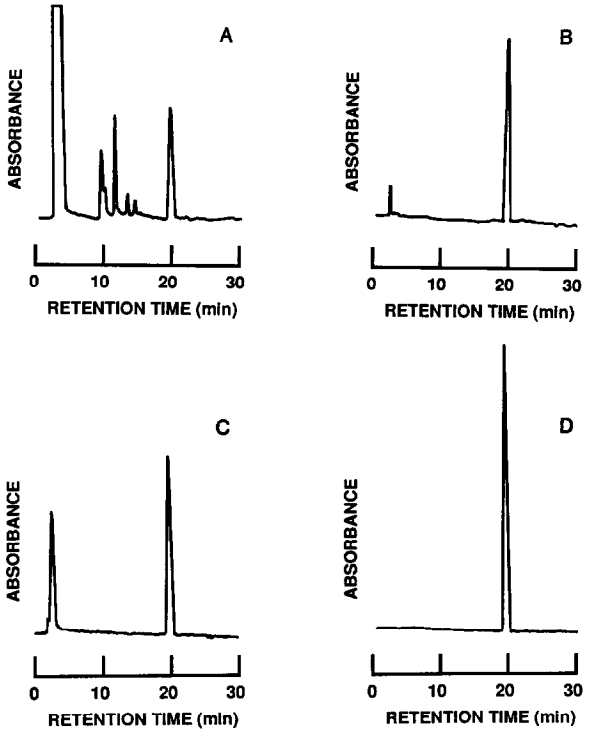


Figure 1. High performance liquid chromatography of crude and purified a-factor. Panel A: crude a-factor from the solid-phase synthesis. Panel B: Purified a-factor from the solution-phase synthesis. Panel C: Purified a-factor from the solid-phase synthesis. Panel D: Coinjection of B and C. The peptides were eluted from a C_{18} reversed phase column using H_2O (0.025% CF_3COOH)/CH_3CN with a linear gradient from 20% CH_3CN to 80% CH_3CN.

(4:1:1); R_f=0.58 and butanol:acetic acid:water:pyridine (15:3:8:10) R_f=0.82 as the eluents. It had an optical rotation $[\alpha]_D$ = 64° (c 0.1, methanol). After a 72 h hydrolysis in 6N HCl the following residues were found Asp(1.06); Gly(1.07); Ala(0.96); Pro(1.01); Tyr(1.03); Val(1.02); Ile(1.80); Phe(1.00); Lys(1.01). The FAB mass spectrum indicated a molecular ion $(M+H)^+$ of 1629.7 (calculated 1629.9) and fragments in agreement with those reported by Anderegg et al. (5). 400 MHz ^{1}H-NMR supported the presence of Trp, trans-trans farnesyl, and the methyl ester.

Biological Activity of Synthetic Pheromone: The activity of the synthetic dodecapeptide was measured using both a growth arrest (halo) and morphogenesis (shmooing) assay. Due to the low solubility of the pheromone in water, it was originally dissolved in either 25% acetic acid or methanol. The methanolic solution was diluted first into 25% acetic acid and then serially diluted 1:2 through distilled water, whereas the acetic acid solution was serially diluted directly 1:2 through water. The endpoint of the Halo assay was 100 pg/spot of pheromone using the acetic acid stock solution, whereas an endpoint of 6 pg/spot was obtained with the methanolic

solution. The difference in endpoints may reflect the greater solubility of the hydrophobic pheromone in the organic solvent. Noticeable shmooing occurred at concentrations as low as 40-80 ng/ml using the acetic acid solution. This activity compares favorably with the values observed for the biological pheromone by Anderegg (25-125 pmol/ml; 40-200 ng/ml) for the same yeast strain using a growth arrest assay (5), and is indicative of a highly potent pheromone. The solid-phase pheromone had activity comparable to the solution-phase pheromone in the halo assay.

DISCUSSION

The results presented in this paper confirm that the synthetic lipopeptide; YIIKGVFWDPA(S-farnesyl)OCH_3, is identical in physical, chemical and biological properties to the natural peptide a2 recently identified by Anderegg et al.(5). The efficient synthesis of this molecule and the ready availability of mg quantities of the lipopeptide should stimulate investigations of the biosynthesis of the pheromone and its mechanism of action. Finally, the synthetic route reported herein is readily extendable to analogs of the pheromone both at the carboxyl terminus and the alkyl side chain on the cysteine sulfur. Such analogs are needed to understand the relationship between the structure of the a-factor and its biological activity.

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REFERENCES

1. Levi, J.D. (1956) Nature 177, 753-754.
2. Stotzler, D., Kiltz, H., and Duntze, W. (1956) Eur. J. Biochem. 69,397-400.
3. Masui, Y., Chino, N., Sakakibara, S., Tanaka, T., Murakami. T., and Kita, H., (1977) Biochem. Biophys. Res. Commun. 78, 534-538.
4. Ciejek, E., Thorner, J., and Geier, M., (1977) Biochem. Biophys. Res. Commun. 78, 952-961.
5. Anderegg, R.J., Betz, R., Carr, S.A., Crabb, J.W., and Duntze, W. (1988) J. Biol. Chem. 263, 18236-18240.
6. Sakagami, Y., Isogai, A., Suzuki, A., Tamura, S., Tsuchiya, E., and Fukui, S., (1978) Agric. Biol. Chem. 42 1093-1094.
7. Kamiya, Y., Sakurai, A., Tamura, S., Takahashi, N., Tsuchiya, E., Abe, K.,and Fukui, S., (1979) Agric. Biol. Chem. 43, 363-369.
8. Fuller, R.S., Sterne, R.E., and Thorner, J. (1988) Ann. Rev. Physiol. 50, 345-362
9. Brake, A.J., Brenner, C., Najarian, R., Laybourn, P., and Merryweather, J. (1985) in Current Communications in Molecular Biology, (M.J. Gething Ed), pp.103-108 Cold Spring Harbor Laboratory, New York .
10. Castro, B., Dormoy, J.R., Evin, G. and Selve, C. (1975) Tetrahedron lett., 14, 1219-1222
11. Betz,R., Crabb, J.W., Meyer, H.E., Wittig, R., and Duntze, W. (1987) J. Biol. Chem. 262, 546-548.