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Synthesis of a-factor peptide from *Saccharomyces cerevisiae* and photoactive analogues via Fmoc solid phase methodology

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ABSTRACT

a-Factor from *Saccharomyces cerevisiae* is a farnesylated dodecapeptide involved in mating. The molecule binds to a G-protein coupled receptor and hence serves as a simple system for studying the interactions between prenylated molecules and their cognate receptors. Here, we describe the preparation of **a**-factor and two photoactive analogues via Fmoc solid-phase peptide synthesis using hydrazinobenzoyl AM NovaGel™ resin; the structure of the synthetic **a**-factor was confirmed by MS–MS analysis and NMR; the structures of the analogues were confirmed by MS–MS analysis. Using a yeast growth arrest assay, the analogues were found to have activity comparable to **a**-factor itself.

1. Introduction

In the budding yeast, *Saccharomyces cerevisiae*, mating between the two haplotypes involves the participation of two secreted peptide pheromones.[1] Each of these molecules binds to a specific G-protein coupled receptor and activates a signal transduction cascade resulting in a series of complex cellular events. One of these peptides is **a**-factor (**1**, Fig. 1), a dodecapeptide that incorporates farnesylcysteine methyl ester as its C-terminal residue.[2] The **a**-factor/receptor system is a useful model for studying the interactions between prenylated molecules and proteins.[3] Due to their widespread occurrence,

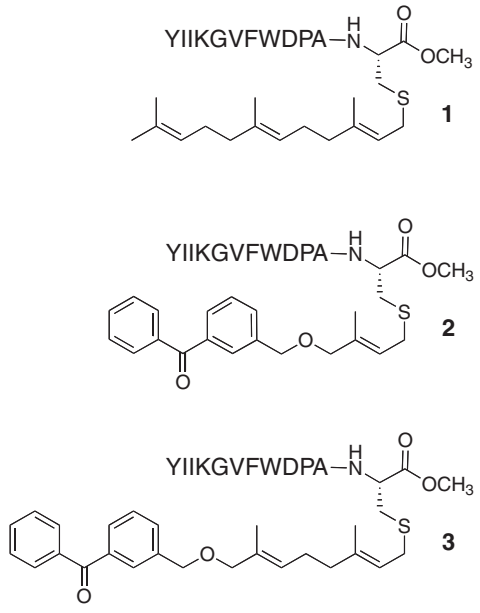

Figure 1. Structures of **a**-factor (**1**) and photoactive analogues with 5-carbon (**2**) and 10-carbon (**3**) isoprenoid spacers.

Abbreviations: Boc, *tert*-butyloxycarbonyl; Br-BC5, [(*E*)-(3-((4-bromo-2-methylbut-2-enyloxy)methyl)phenyl)-(phenyl)methanone]; Br-BC10, [(3-(((2*E*,6*E*)-8-bromo-2,6-dimethylocta-2,6-dienyloxy)methyl)-phenyl)(phenyl)methanone]; *t*-Bu, *tert*-butyl; CLEAR, cross-linked ethoxylate acrylate resin; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene) ethyl; DI, deionized; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; FA, formic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Fr, farnesyl; Fr-Br, farnesyl bromide; HPLC, high pressure liquid chromatography; OAc, acetate; PBS, phosphate buffered saline; PEG, polyethylene glycol; RP-HPLC, reversed-phase high pressure liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TFP, tetrafluorophenyl; Trt, trityl.

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their participation in cellular signaling and their pivotal role in numerous diseases, prenylated proteins have been the focus of intense study within the last 15 years.[4,5] While initially prenylation was thought to function only as a membrane targeting element, it is now clear that this lipid modification is also involved in directly mediating certain protein–protein interactions.[6] The binding of **a**-factor to its receptor is one example of this latter phenomenon.[7]

Given the membrane bound nature of Ste3p, the **a**-factor receptor, photoaffinity labeling is an attractive method to probe the interaction between the protein and the pheromone. Several laboratories have developed analogues of the farnesyl moiety that incorporate photoactivatable groups into the isoprenoids themselves, allowing specific interactions between prenyl groups and their cognate protein receptors to be studied; those molecules include diazotrifluropropionates[8–16], benzophenones[17–27] and aryl azides.[28,29] Thus, we envisioned that **a**-factor analogues suitably functionalized with photoactive farnesyl groups would be useful for this purpose and accordingly, we sought to prepare such molecules via solid-phase peptide synthetic methods.

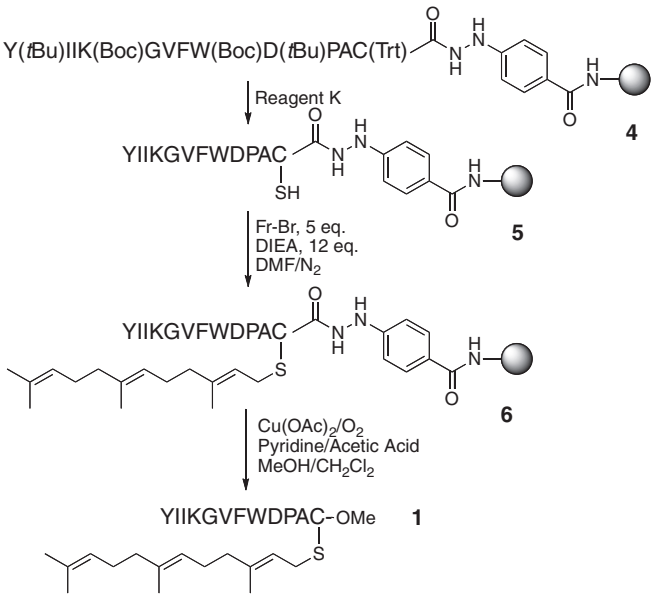
Chemical synthesis using the Merrifield Boc/Bzl protecting group strategy[30] has been used extensively for the preparation of **a**-factor and related analogues.[31–34] That strategy has been the method of choice due to the presence of the C-terminal ester. Recently, Waldmann and co-workers developed a method for generating C-terminal methyl esters based on a hydrazine-containing resin.[35,36] In their approach, synthesis begins with attachment of the C-terminal residue to hydrazinobenzoyl AM NovaGel™ resin via a hydrazide linkage. Following standard synthetic protocols involving iterative cycles of amide bond formation and deprotection, the completed resin-bound fully protected peptide is then subjected to Cu(I) mediated oxidation of the hydrazide linkage. This transformation yields a diazo-ester intermediate that undergoes spontaneous alcoholysis; global side-chain deprotection affords the desired C-terminal ester product. An important feature of this strategy is that it is fully compatible with the Fmoc/t-Bu protecting group scheme[37] whose popularity has increased due to the elimination of the need to work with HF in the final deprotection/resin cleavage step. Given that the synthesis of cysteine-containing peptides can be problematic due to the possibility of epimerization and b-elimination,[38] the well precedented hydrazide strategy appeared attractive. While other methods such as the xanthenyl side-chain anchoring strategy developed by Barany et al. have great potential for the synthesis of peptides containing C-terminal cysteine residues,[39] the resin required to implement that strategy is not commercially available. Thus, we elected to explore the use of hydrazine-containing resin available from Novabiochem for **a**-factor synthesis.

In the work reported here, we first describe the preparation of **a**-factor via Fmoc SPPS using hydrazinobenzoyl resin; the structure of the material prepared by this alternative synthetic route was confirmed by detailed MS–MS analysis. Using a yeast growth arrest assay, the synthetic material was found to be equipotent compared with the **a**-factor prepared previously[31–34] The resulting method was then used to prepare two **a**-factor analogues containing photoactive benzophenone groups incorporated within the isoprenoid moiety. Using the same growth arrest assay, the photoactivatable analogues were found to have activity comparable to that of **a**-factor itself.

2. Results and discussion

2.1. Attempted synthesis of a-factor peptide precursor by oxidative resin cleavage of an unprotected peptide

The syntheses of **a**-factor (**1**) and benzophenone analogues **2** and **3** present several challenges. First, the all *trans* double bonds of the prenyl groups are not stable to the acidic conditions necessary to remove standard *t*-butyl-based side-chain protecting groups used in Fmoc chemistry;[40] exposure of isoprenoids to acidic conditions typically leads to complex mixtures of solvolyzed and rearranged products. Next, because the C-terminal methyl ester is necessary for activity, standard amide and acid SPPS resin linkages are not suitable for use in this synthesis. Recent reports by Waldmann et al.[41–43] on the synthesis of lipidated peptides that contain a C-terminal methyl ester by oxidative cleavage of peptides from hydrazinobenzoyl AM NovaGel™ resin[44] offered a possible way forward. We envisioned a synthetic route in which all steps would be done while the peptide was still attached to the solid support (Scheme 1), which would minimize losses from handling and from successive preparative HPLC purifications. In this reaction sequence, after chain assembly by standard Fmoc-based SPPS, the acid labile side-chain protecting groups in the fully protected peptide **4** are first removed by Reagent K[45] before alkylation of cysteine by farnesyl bromide. In the last step, oxidative cleavage of alkylated peptide **6** would produce **a**-factor (**1**). Unfortunately, after oxidative cleavage a complex mixture of products was observed in which only a trace of desired product **1** could be found (see Fig. 2). It is possible that unprotected tryptophan is not stable to these oxidative conditions. Also, in the many examples of oxidative cleavage published by Waldmann and co-workers, the side-chains of trifunctional amino acids are generally protected during this reaction.[41–43] It is possible that unprotected side-chains chelate copper and interfere with the cleavage reaction or undergo oxidation themselves.



Scheme 1. Initial strategy for the synthesis of **a**-factor peptide precursor by oxidative resin cleavage of an unprotected peptide.

2.2. Successful approach of a-factor and analogues via a fully protected peptide

To circumvent the above problems, we decided to attempt oxidative cleavage on a fully protected peptide sequence (Scheme 2). Before resin cleavage, the amino terminus of the peptide was protected with the Boc group by treatment with di-*tert*-butyl dicarbonate in the presence of base to yield an on-resin fully protected peptide **7**. Oxidative cleavage by Cu(I) with methanol as a cosolvent furnished fully protected methyl ester **8**, which was treated with Reagent K to cleave the acid labile protecting groups. We were pleased to find only one main product by HPLC analysis that corresponded to desired peptide **9**. This result

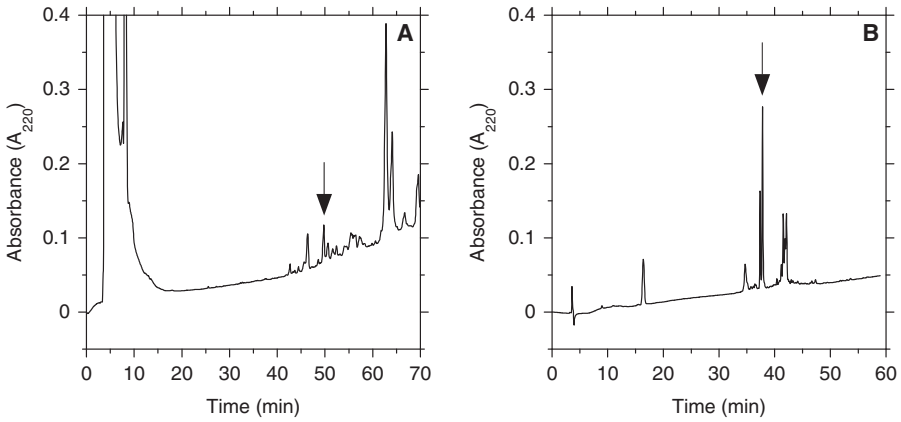
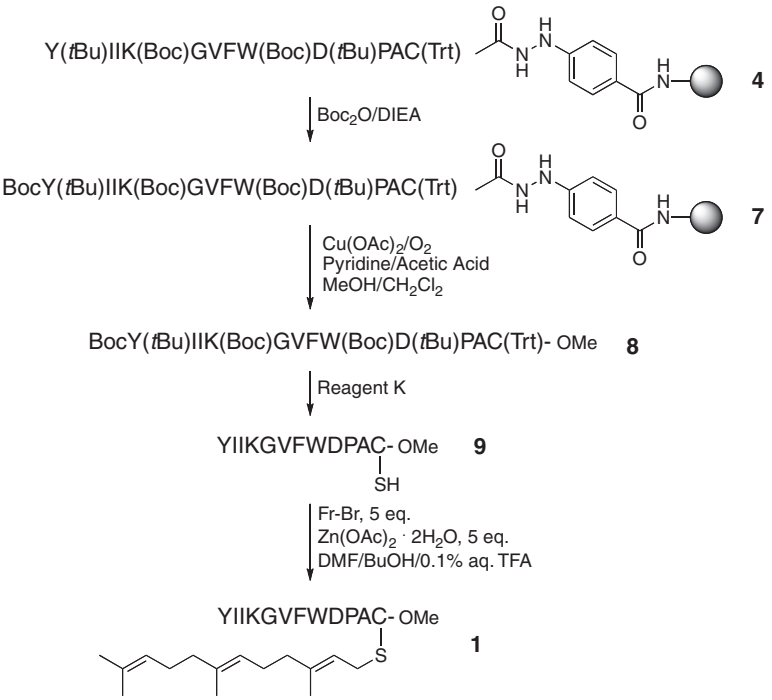


Figure 2. HPLC of crude reaction mixtures from the preparation of **a**-factor after oxidative resin cleavage Panel A: HPLC of crude reaction mixture of **1** after oxidative cleavage of **6** as described in Scheme 1. The arrow indicates the peak containing **1**. Panel B: HPLC of crude reaction mixture of **9** after oxidative cleavage and Reagent K treatment as shown in Scheme 2. The arrow indicates the peak containing **9**.



Scheme 2. Successful strategy for the synthesis of **a**-factor peptide (**1**) precursor by oxidative resin cleavage of a fully protected peptide.

supported our hypothesis that a fully protected **a**-factor sequence was necessary for successful oxidative cleavage. Alkylation of the cysteine thiol of **9** with farnesyl bromide, catalyzed by Zn^{2+}[40] gave **a**-factor (**1**) (Scheme 2). Benzophenone analogues **2** and **3** were obtained by reaction of **9** with the corresponding allylic bromides under the same alkylation conditions.

It is worth noting that yields of alkylated products **1**, **2**, and **3**, appear low (8–19%) when calculated based on the masses of the starting material and products; accordingly, we investigated this by resynthesizing **1**. However, in this second synthesis, the amount of peptide **9** used was determined by DTNB titration while the amount of product (**1**) was quantified via its tryptophan chromophore. As was seen in the first synthesis of **1**, HPLC analysis of the alkylation reaction of **9** with farnesyl bromide (Fig. 3) revealed nearly complete consumption of **9** and relatively clean conversion to **1** with few side products formed, When the yield of **1** from this reaction was calculated from the masses of the starting material and product, the value was determined to be 19%. However, the yield obtained via DTNB/UV analysis was calculated to be 65%. This latter value is comparable to alkylation yields obtained using the $Zn(OAc)_2$ method on related peptides.[7,46–48] In general, it is likely that the lower yields of the peptide products quantified by mass reflect the presence of H_2O and/or TFA salts as well as uncertainties inherent in the measurement of small (~1–2 mg) masses.

2.3. Mass spectrometric analysis of a-factor and two related photoactive analogues

Since a new synthetic route was used to obtain **a**-factor and the two photoactive analogues, the structures of these peptides were examined in detail by mass spectrometry. ESI-MS–MS spectra of **1**, **2**, and **3** are shown in Figures 4–6. Similar fragmentation was observed for all three peptides. In general, the results observed are consistent with MS–MS data previously reported for **a**-factor by

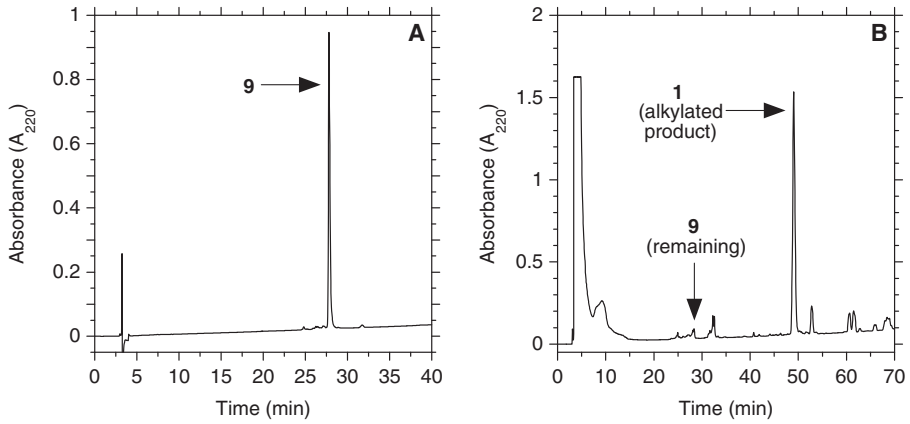


Figure 3. HPLC analysis of starting material and crude reaction mixture from the alkylation of pure **9** to yield **a**-factor (**1**). Panel A: HPLC of purified starting material **9**. Panel B: HPLC of crude alkylation reaction mixture containing **9** and farnesyl bromide in the presence of $Zn(OAc)_2$ to yield **1**.

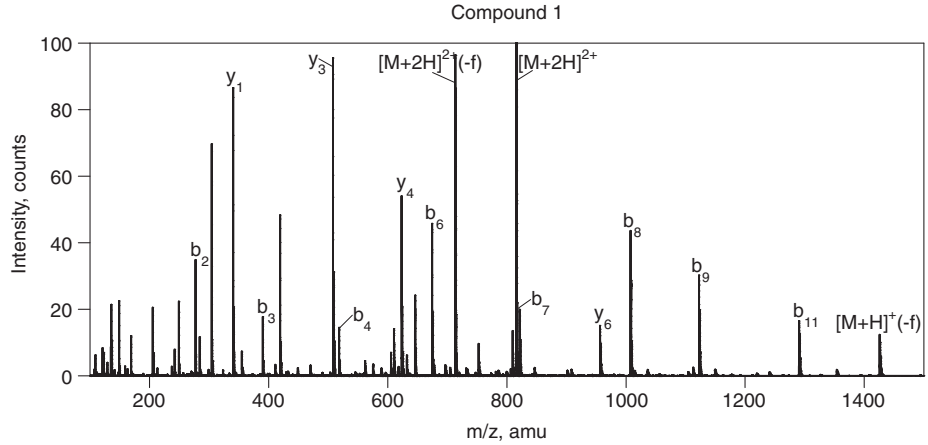


Figure 4. ESI-MS-MS analysis of **a**-factor (**1**) produced by Fmoc SPPS. -f: loss of farnesyl (loss of $C_{15}H_{25}$).

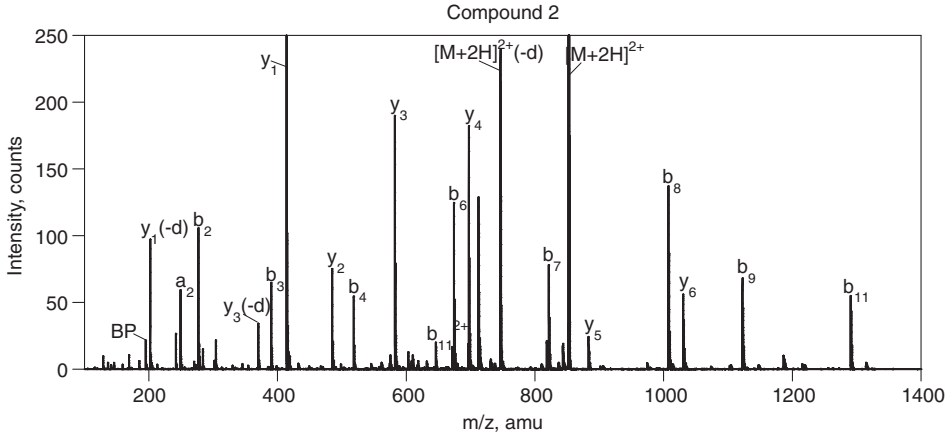


Figure 5. ESI-MS-MS analysis of **a**-factor analogue **2** incorporating a photoactive isoprenoid with a 5-carbon spacer. BP: benzophenone fragment, (3-benzoylphenyl)methylium, ($C_{14}H_{11}O^+$); -d: loss of (3-(hydroxymethyl)phenyl)(phenyl)menthanone ($C_{14}H_{13}O_2$).

Anderegg et al.[2] In that work, MS–MS data was obtained by FAB ionization in lieu of ESI as was employed here. In their work, the fragment ion (m/z = 1425.7) corresponding to the singly-charged parent ion with loss of the isoprenoid group was identified, along with several b-type ions. Interestingly, they reported the $[M+H]^+$ parent ion (m/z = 1629.9) as the most abundant species in the FAB-MS and fragmented that ion to obtain their MS–MS data; in contrast, we observed the doubly-charged $[M+2H]^{2+}$ parent ion (m/z = 815.5) as the most abundant ion in the ESI-MS. Accordingly, fragmentation was performed on that species for MS–MS analysis and yielded primarily b- and y-type ions (see Table 1 in Supplementary data for complete listings and assignments). Fragmentation corresponding to cleavage of the thioether bond linking the isoprenoid moiety to the cysteinyl thiol was observed in the MS–MS of **a**-factor, **1**, and

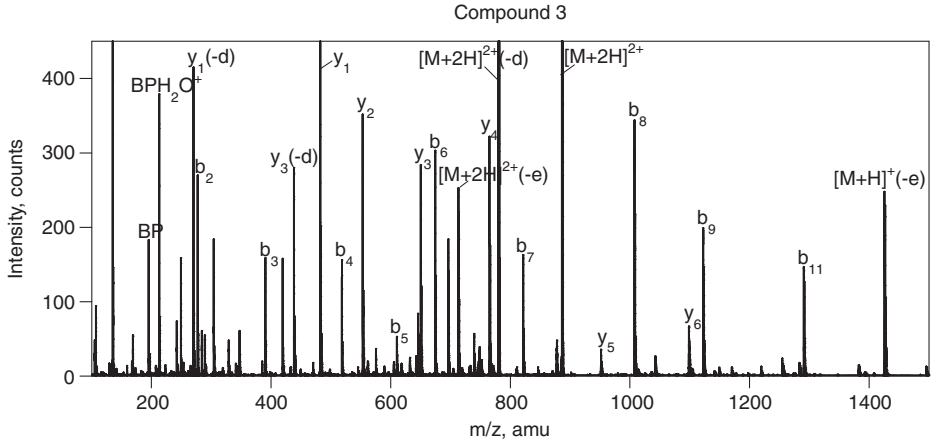


Figure 6. ESI-MS-MS analysis of **a**-factor analogue **3** incorporating a photoactive isoprenoid with a 10-carbon spacer. BP: benzophenone fragment, (3-benzoylphenyl)methylium, ($C_{14}H_{11}O^+$); BPH_2O^+: (3-benzoylbenzyl)-oxonium, ($C_{14}H_{13}O_2^+$); -d: loss of (3-(hydroxymethyl)phenyl)(phenyl)menthanone ($C_{14}H_{13}O_2$); -e: loss of (3-(((2E,6E)-2,6-dimethylocta-2,6-dienyloxy)methyl)phenyl)(phenyl)methanone, (loss of $C_{24}H_{28}O_2$).

the related analogue, **3**; this type of fragmentation has also been previously noted in analyses of **a**-factor via FAB-MS.[49] Both the singly and doubly-charged parent ions with loss of isoprenoid (m/z = 1425.7 and m/z = 713.4) (Figs. 4 and 6) were observed; this was not observed for analogue **2**. Cleavage of the ether bond linking the benzophenone group to the isoprenoid spacer was noted in the MS–MS of both **2** and **3** indicated by the doubly-charged parent ions (m/z = 746.4 and m/z = 780.4 for **2** and **3**, respectively) (Figs. 4 and 5) and a few y-type ions showing loss of (3-(hydroxymethyl)phenyl)(phenyl)menthanone (–BPH_2O). A charged BPH_2O^+ fragment (m/z = 213.1) and the related benzophenone (BP) fragment (3-benzoylphenyl)methylium (m/z = 195.1) were also observed for both **2** and **3**. In aggregate, these data clearly confirm the successful preparation of **a**-factor and related analogues.

2.4. ^{1}H NMR analysis of a-factor (1) produced and photoactive analogues

While the MS–MS experiments described above provided strong evidence for the formation of **a**-factor and the desired photoactive analogues, we felt it would be useful to obtain ^{1}H NMR data on **1**, prepared via the new route reported here. Accordingly, a 2D ^{1}H–1H TOCSY NMR spectrum was obtained; a portion of the spectrum from the fingerprint region is shown in Figure 7. Comparison of this spectral data with data previously reported for **a**-factor (prepared via a different route employing Boc/Bzl protection) shows excellent agreement. Hence, these results provide compelling corroborating evidence that the methodology presented here can be used to produce **a**-factor.

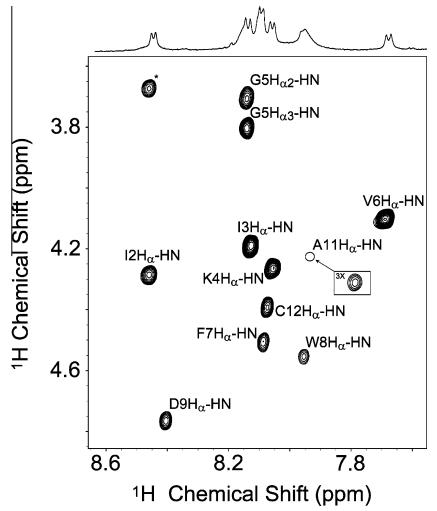


Figure 7. Fingerprint region from a 2D ^{1}H–^{1}H TOCSY NMR spectrum of **a**-factor (**1**) in DMSO-d_6. Assignments are indicated adjacent to each cross peak. A peak arising from exchange of the amide proton of Ile 2 with residual H_2O is marked with an asterisk in the spectrum. The peak corresponding to Ala 11 could be detected at a threefold lower contour level as shown in the inset.

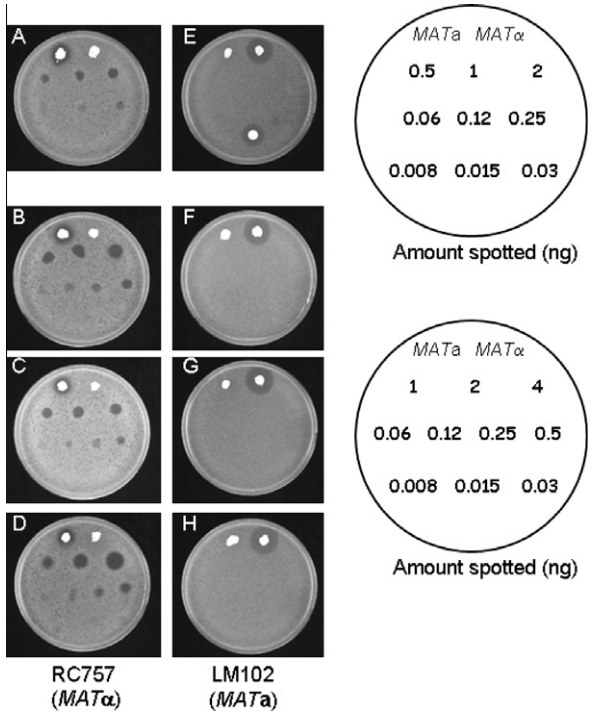


Figure 8. Biological assay of pheromones. Growth arrest in response to pheromone was determined for the **a**-factor responsive strain RC757 (A–D) or the α-factor responsive strain LM102 (E–H). The upper template indicates the amount of control synthetic **a**-factor that was spotted on plates A and E. A disk impregnated with α-factor (0.2 μg) was applied to the bottom of the plate E. Compounds were applied to the remaining plates as indicated in the lower template: B and F, synthetic **a**-factor (**1**), C and G **3**, D and H, **2**. At the top of each plate (A–H) cells secreting **a**-factor (*MAT***a**) or α-factor (*MAT***α**) were applied to the lawn as indicated in the templates.

2.5. Biological assay of synthetic a-factor and photoactive analogues

To verify that the **a**-factor produced using the new synthetic route described here retained full biological activity, it was evaluated in a yeast growth arrest assay. Cells expressing the *STE3* **a**-factor receptor protein (*MAT*α cells) undergo growth arrest in the presence of **a**-factor peptide. In this assay, a plate containing a lawn of *MAT*α *S. cerevisiae* cells (strain RC757) was treated with different amounts of **a**-factor. If the cells detect **a**-factor, a clear zone of inhibition will develop in the region where the compound is applied. Several controls were included in this experiment. First, *S. cerevisiae* cells which secrete biologically-synthesized **a**-factor (X2180-1A) or α-factor (X2180-1B) were applied to the lawn. A clear zone of inhibition formed around the **a**-factor secreting cells but not around those secreting α-factor (Fig. 8A–D) indicating that the RC757 cells respond specifically to **a**-factor. A previously characterized, synthetic **a**-factor with wild-type potency[31–34] also stimulated growth arrest with an endpoint of 0.12 ng; the synthetic material produced in this study yielded an identical endpoint (see Fig. 8A and B). Next the biological activity of the **a**-factor analogues containing the photoactive isoprenoids was evaluated using the same assay. Analogue **2** containing the shorter isoprenoid spacer gave an endpoint of 0.25 ng (see Fig. 8D) while analogue **3** functionalized with the longer isoprenoid spacer gave an endpoint of 0.50 ng (see Fig. 8C). To ensure that the observed growth arrest was not simply due to general toxicity, control experiments were performed using a *MAT***a** strain (LM102), which expresses the α-factor receptor and thus does not respond to **a**-factor.

Using LM102, no growth arrest was observed in response to cell-secreted **a**-factor, but growth arrest was observed in response to cell-secreted α-factor (Fig. 8E–H) and in response to a disc impregnated with synthetic α-factor (Fig. 8E), thus indicating that these cells were sensitive to their cognate pheromone. The LM102 cells did not yield a zone of growth inhibition in response to any of the **a**-factor peptides indicating that compounds **1**, **2**, and **3** are not toxic and are mating type specific (Fig. 8E–H). Thus, based on the endpoint determinations reported above, the **a**-factor produced via this new route is equipotent with naturally-derived material and the photoactive analogues retain most (25–50%) of the biological activity of the parent **a**-factor pheromone.

3. Conclusion

An efficient synthesis of **a**-factor peptide from *S. cerevisiae* using Fmoc solid phase methods is reported here. This synthesis has been used to produce **a**-factor as well as two benzophenone-containing analogues. The photoactive probes retain near-wild type levels of activity in a yeast growth arrest assay. The simplified route to **a**-factor analogues described herein should facilitate investigation of **a**-factor receptor and other proteins that interact with prenylated peptides and proteins.

4. Experimental

4.1. General

All solvents were of HPLC grade. DIEA and TFA were of Sequalog/peptide synthesis grade from Fisher. Hydrazinobenzoyl AM NovaGel™ resin was obtained from Novabiochem. Farnesyl bromide (>95% *E,E*) was obtained from Sigma–Aldrich. Standard Fmoc/HCTU chemistry was used for SPPS of the peptides. Vydac 218TP54 and 218TP1010 columns were used for analytical and preparative RP-HPLC, respectively. All analytical and preparative RP-HPLC solvents, water and CH_3CN, contained 0.10% TFA. Retention times (t_R) are based on 1% per minute linear gradients starting at 0% B (CH_3CN). The term 'overall yield' includes chain assembly, further on-resin or in solution conversions, Reagent K cleavage, and RP-HPLC purification steps. The benzophenone-containing isoprenoids Br-BC5 [(*E*)-(3-((4-bromo-2-methylbut-2-enyloxy)methyl) phenyl)(phenyl)-methanone] and Br-BC10 [(3-(((2*E*,6*E*)-8-bromo-2,6-dimethylocta-2,6-dienyloxy)methyl)phenyl)-(phenyl)methanone] were prepared as previously described.[21,26]

4.2. Chemical synthesis

4.2.1. YIIKGVFWDPAC-OCH_3 (9)

The linear sequence, Y(*t*-Bu)IIK(Boc)GVFW(Boc)D(*t*-Bu)-PAC(Trt)-hydrazide-resin (**4**) was synthesized on 4-Fmoc-hydrazino–benzoyl AM NovaGel® resin on the ABI 433 peptide synthesizer by standard FastMoc chemistry. To protect the N-terminus with the Boc group, 11 μmol of peptide resin was swollen in DMF. Di-*tert*-butyl dicarbonate (44 μmol, 96 mg) and DIEA (88 μmol, 153 μL) were added, and the reaction tumbled for 4 h. The resin was washed with DMF and CH_2Cl_2 and was dried in vacuo to yield **7**. To the resin was then added Cu(II)acetate (11 μmol, 20 mg), and the resin was then swollen with 2 mL anhydrous CH_2Cl_2 under N_2. Glacial acetic acid (6.0 mmol, 400 μL), anhydrous pyridine (3.5 mmol, 280 μL), and anhydrous methanol (21 mmol, 880 μL) were added to the resin. Oxygen was bubbled through the reaction for about 1 min and the reaction was agitated under an oxygen atmosphere for 2.5 h. The resin was removed by filtration and washed with CH_2Cl_2 and methanol. Most of the solvents were removed by rotary evaporation, and the oily residue was lyophilized from dioxane to produce crude **8**. The solid that resulted was treated with freshly prepared Reagent K (8 mL) for 30 min to remove protecting groups. The peptide was precipitated by the addition of 80 mL ether. After centrifugation to form a pellet, which was washed twice with ether, the peptide was dissolved in about 5 mL methanol, filtered to remove copper salts, and purified by RP-HPLC. Yield 21.4 mg (14% from 110 μmol peptide resin), purity by HPLC: 90%. ESI-MS: calcd: 1424.7, found: 1424.6. t_R = 37.6 min.

4.2.2. YIIKGVFWDPAC(Fr)-OCH_3 (1)

YIIKGVFWDPAC-OCH_3 (**9**, 10 mg, 7.0 μmol) was dissolved in DMF/1-butanol (2:1 v/v, 6.0 mL). Farnesyl bromide (35 μmol, 5 equiv, 10 μL) was added to the reaction and an HPLC analysis performed. Then, $Zn(OAc)_2{\cdot}2H_2O$ (35 μmol, 5 equiv, 8 mg) dissolved in 0.10% aqueous TFA (2.0 mL) was added to initiate the alkylation reaction. The reaction was monitored by RP-HPLC, and once judged complete (typically 1 h), the solution was filtered, and purified by RP-HPLC. Yield 1.0 mg (9%), purity by HPLC: 93%. ESI-MS: calcd: 1628.9, found: 1628.8. t_R = 58.7 min. In a second synthesis, **9** (12.6 mg, 8.8 μmol) was dissolved in DMF/1-butanol (2:1 v/v, 8.0 mL). It should be noted (see below) that subsequent DTNB titration[50] of the sample of **9** used for this reaction indicated that the true peptide content of the material was only 5.8 μmol due to the presence of water and/or TFA salts. Farnesyl bromide (44 μmol, 5 equiv, 12 μL) was added to the reaction and an HPLC analysis was performed. Then, $Zn(OAc)_2{\cdot}2H_2O$ (44 μmol, 5 equiv, 9.6 mg) dissolved in 0.10% aqueous TFA (1.0 mL) was added to initiate the thiol alkylation. The reaction was monitored by RP-HPLC and once judged complete (within 1 h), the solution was filtered, and purified by RP-HPLC. The mass of the product was 2.8 mg (1.7 μmol) but UV measurement based on tryptophan absorbance (e_{280} = 6990 M^{-1} cm^{-1})[51] indicated 3.8 μmol present. Using these numbers, yields of 19% yield based on mass and 65% based on DTNB/UV measurements were calculated. A purity of 98% was determined by HPLC. ESI-MS: calcd: 1628.9, found: 1628.8. t_R = 58.7 min.

4.2.3. YIIKGVFWDPAC(BC5)-OCH_3 (2)

YIIKGVFWDPAC-OCH_3 (**9**, 10 mg, 7.0 μmmol) was dissolved in DMF/1-butanol (2:1 v/v, 6.0 mL). To purify Br-BC5 [(*E*)-(3-((4-bromo-2-methylbut-2-enyloxy)methyl)phenyl)(phenyl)methanone][21] (35 μmol, 12 mg) before reaction, it was dissolved in 0.50 mL of DMF and loaded onto a C18 SepPack® column that had been equilibrated with 5% CH_3CN in aq. 0.10% TFA. The column was washed with 10 mL of the 5% CH_3CN solution, followed by 10 mL of 30% CH_3CN. The purified bromide, Br-BC5, was then eluted from the column with 5.0 mL DMF directly into the reaction flask that contained the peptide. Then, $Zn(OAc)_2{\cdot}2H_2O$ (35 μmol, 5 equiv, 8 mg) dissolved in 0.10% aqueous TFA (2.0 mL) was added to initiate the alkylation reaction. The reaction was monitored by RP-HPLC, and once judged complete (typically 1 h), the solution was filtered, and purified by RP-HPLC. Yield 1.3 mg (8% by mass, note comments above concerning **1**), purity by HPLC: 90%. ESI-MS: calcd: 1702.8, found: 1702.8. t_R = 50.1 min.

4.2.4. YIIKGVFWDPAC(BC10)-OCH_3 (3)

YIIKGVFWDPAC-OCH_3 (**9**, 10 mg, 7.0 μmol) was dissolved in DMF/1-butanol (2:1 v/v, 6.0 mL). To purify Br-BC10 [(3-(((2*E*,6*E*)-8-bromo-2,6-dimethylocta-2,6-dienyloxy)methyl)phenyl)(phenyl)-methanone][26] (28 μmol, 4 equiv, 12 mg) before reaction, it was dissolved in 0.50 mL of DMF and loaded onto a C18 SepPack® column that had been equilibrated with 5% CH_3CN in aq. 0.10% TFA. The column was washed with 10 mL of the 5% CH_3CN solution, followed by 10 mL of 30% CH_3CN. Br-BC10 was then eluted from the column with 5.0 mL DMF directly into the reaction flask that contained the peptide. Then, $Zn(OAc)_2{\cdot}2H_2O$ (35 μmol, 5 equiv, 8 mg) dissolved in 0.10% aqueous TFA (2.0 mL) was added to initiate the alkylation reaction. The reaction was monitored by RP-HPLC, and once judged complete (typically 1 h), the solution was filtered, and purified by RP-HPLC. To separate the product from unreacted Br-BC10 it was necessary to use a preparative C4 column. Yield 1.7 mg (14% by mass, note comments above concerning **1**), purity by HPLC: 97%. ESI-MS: calcd: 1770.9, found: 1770.8. t_R = 45.5 min.

4.3. Mass spectrometric analysis of a-factor, 1, and photoactive analogues 2 and 3

A small amount of lyophilized powder from **1**, **2** or **3** was dissolved in 10 μL of 0.1% TFA/CH_3CN and further diluted 1:50 in 0.1% formic acid/50% CH_3CN prior to MS analysis. MS was performed using 10 μL injections and 5 min data acquisition on a QSTAR1 mass spectrometer. The doubly-charged $[M+2H]^{2+}$ species (m/z = 815.5; m/z = 852.4; m/z = 886.4 for **1**, **2**, and **3**, respectively) were most abundant and were selected for subsequent MS–MS analysis. MS results were evaluated using Analyst software and sequenced using UCSF Protein Prospector v 5.5.0.

4.4. ^{1}H NMR analysis of a-factor, 1

For NMR analysis, **a**-factor (**1**) was dissolved in DMSO-d_6 to a concentration of 1.6 mM. A TOCSY experiment was recorded at 25 °C on a Varian Inova spectrometer operating at a 600 MHz proton frequency. 4 scans were acquired with 2048 complex points in the direct dimension and 256 points in the indirect dimension. Spectral widths of 12000 Hz were used in both dimensions and a spin-lock field of 7000 Hz was applied for 75 ms. Data was zero-filled to a final matrix size of 2048 × 2048 points after Fourier transformation. Resonances were assigned based on the previous assignment by Gounarides et al.[52]

4.5. Biological assays

Saccharomyces cerevisiae strains RC757 (*MAT*α *sst2-1 rme1 his6 met1 can1 cyh2*) and LM102 (*MAT*a, *bar1*, *his4*, *leu2*, *trp1*, *met1*, *ura3*, *FUS1-lacZ*::*URA3*, *ste2-dl*) were used to test biological activity as previously described.[34] The strain LM102 bears the plasmid pBEC2[53] encoding Ste2p, the α-factor receptor. RC757 cells were cultured in YEPD (1% yeast extract, 2% peptone, 2% dextrose) while LM102 cells were cultured in MLT[54] to ensure maintenance of the plasmid. Cells were grown overnight at 30 °C with shaking in liquid medium. For use in the growth arrest assay, cells were harvested by centrifugation (1000 *g*), washed twice with sterile water, and resuspended to a final concentration of 1×10^6 cells/mL in water. The cell suspension (1 mL) was combined with 3 mL Noble agar (1.1% in water) and overlaid onto solid medium (YEPD or MLT containing 2% agar). The peptides were dissolved in MeOH (10 ng/μL) and diluted in 0.5% bovine serum albumin (BSA) to a final concentration of 0.8 ng/μL, then serially diluted in 0.5% BSA to generate solutions of the desired concentrations. Five microliters of each dilution was spotted onto the overlay containing RC757 or LM102 cells. The plates were spotted in triplicate, and incubated overnight at 30 °C. The experiment was repeated twice with similar results. The endpoint of the assay was determined to be the lowest concentration at which a clear zone of inhibition, which indicates growth arrest, was observed. Wild type *S. cerevisiae* strains X2180-1A (*MAT*a) and X2180-1B (*MAT*α) were cultured on YEPD medium and used as endogenous sources for **a**-factor and **α**-factor, respectively. The peptide WHWLQLKPGQPNle[12]Y[55] impregnated onto sterile paper disks was used as a source of synthetic α-factor.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.006.

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