Scanning Alanine Mutagenesis and De-peptidization of a Candida albicans Myristoyl-CoA:Protein N-Myristoyltransferase Octapeptide Substrate Reveals Three Elements Critical for Molecular Recognition*  

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Candida albicans produces a single myristoyl-CoA:protein N-myristoyltransferase (Nmt) that is essential for its viability. An ADP-ribosylation factor (Arf) is included among the few cellular protein substrates of this enzyme. An octapeptide (GLYASKLS-NH₂) derived from a N-terminal Arf sequence was used as the starting point to identify elements critical for recognition by the acyltransferases’s peptide-binding site. In vitro kinetic studies, employing purified Nmt and a panel of peptides with single Ala substitutions at each position of GLYASKLS-NH₂, established that its Gly1, Ser5, and Lys6 residues play predominant roles in binding. AlyASKLS-NH₂ was found to be an inhibitor competitive for peptide (K_i = 15.3 ± 6.4 μM) and noncompetitive for myristoyl-CoA (K_i = 31.2 ± 0.7 μM). A survey of 26 derivatives of this inhibitor, representing (i) a complete alanine scan, (ii) progressive C-terminal truncations, and (iii) manipulation of the chemical-physical properties of its residues 1, 5, and 6, confirmed the important stereospecific requirements for the N-terminal amine, the β-hydroxyl of Ser5, and the ε-amino group of Lys6. Remarkably, replacement of the N-terminal tetrapeptide of ALYASKLS-NH₂ with an 11-aminoundecanoyl group produced a competitive inhibitor, 11-aminoundecanoyl-SKLS-NH₂, that was 38-fold more potent (K_i = 0.40 ± 0.03 μM) than the starting octapeptide. Removal of the primary amine (undecanoyl-SKLS-NH₂), or replacing it with a methyl group (dodecanoyl-SKLS-NH₂), resulted in 26- and 34-fold increases in IC₅₀, confirming the important contribution of the amine to recognition. Removal of LeuSer from the C terminus (11-aminoundecanoyl-SK-NH₂) yielded a competitive dipeptide inhibitor with a K_i (11.7 ± 0.4 μM) equivalent to that of the starting octapeptide, ALYASKLS-NH₂. Substitution of Ser with homoserine, cis-4-hydroxyproline, or tyrosine reduces potency by 3-70-fold, emphasizing the requirement for proper presentation of the hydroxyl group in the dipeptide inhibitor. Substituting D- or L-Lys decreases its inhibitory activity >100-fold, while deletion of the ε-amino group (Nle) or masking its charge (ε-N-acetyl-lysine) produces 4–7-fold attenuations. L-His, but not its D-isomer, can fully substitute for L-Lys, producing a competitive dipeptide inhibitor with similar potency (K_i = 11.9 ± 1.0 μM). 11-Aminoundecanoyl-SK-NH₂ and 11-aminoundecanoyl-SH-NH₂ establish that a simple alkyl backbone can maintain an appropriate distance between three elements critical for recognition by the fungal enzyme’s peptide-binding site: a simple ω-terminal amino group, a β-hydroxyl, and an ε-amino group or an imidazole. These compounds contain one peptide bond and two chiral centers, suggesting that it may be feasible to incorporate these elements of recognition, or functionally equivalent mimics, into a fully de-peptidized Nmt inhibitor.

Candida albicans is a dimorphic, asexual fungus. Ninety percent of patients with acquired immune deficiency syndrome develop C. albicans infections at some point during the course of their disease (1). The few fungicidal drugs currently available have side effects that limit their therapeutic efficacy (2). Long term suppressive or prophylactic therapy with currently effective fungistatic triazoles (3) may hasten the development of drug-resistant strains. Several observations indicate that myristoyl-CoA:protein N-myristoyltransferase (Nmt) may be a good target for the development of a new class of fungicidal agents. C. albicans contains a single NMT gene (4). Metabolic labeling studies indicate that this Nmt covalently attaches [3H]myristate (C14:0) to the N-terminal Gly residues of fewer than 10 cellular proteins during exponential growth on rich media (5). These Nmt substrates include an ADP-ribosylation factor (Arf) (5, 6) and Cga, a protein of unknown function that can complement the growth arrest and mating defects found in strains of Saccharomyces cerevisiae containing a null allele of its G protein α subunit gene, GPA1 (7). Genetic studies have shown that Nmt is essential for viability. A strain of C. albicans was constructed in which one copy of its NMT gene was deleted. A Gly⁴⁴⁷→Asp mutation was introduced into the remaining NMT allele (designated nmt447D). This amino acid substitution produces a marked reduction in the enzyme’s catalytic efficiency at 24 and 37°C, reflecting, in part, a reduction in its affinity for myristoyl-CoA (8). Unlike isogenic NMT/NMT or NMT/nmtΔ strains, nmtΔ/nmt447D cells require myristate for their growth in rich media at 24 and 37°C. Removing myristate results in cell death (8). This lethality correlates with levels of cellular protein N-myristoylation. Arf is completely N-myristoylated, while its N-myristoylation is reduced by the nmtΔ/nmt447D mutation. This suggests that examination of the Nmt-Arf interaction would provide insight into the mechanism of arfinactivation and its role in cell cycle control. A strain of C. albicans containing a null allele of its G protein α subunit gene, GPA1 (7). Genetic studies have shown that Nmt is essential for viability. A strain of C. albicans was constructed in which one copy of its NMT gene was deleted. A Gly⁴⁷→Asp mutation was introduced into the remaining NMT allele (designated nmt447D). This amino acid substitution produces a marked reduction in the enzyme’s catalytic efficiency at 24 and 37°C, reflecting, in part, a reduction in its affinity for myristoyl-CoA (8). Unlike isogenic NMT/NMT or NMT/nmtΔ strains, nmtΔ/nmt447D cells require myristate for their growth in rich media at 24 and 37°C. Removing myristate results in cell death (8). This lethality correlates with levels of cellular protein N-myristoylation. Arf is completely N-myristoylated, while its N-myristoylation is reduced by the nmtΔ/nmt447D mutation. This suggests that examination of the Nmt-Arf interaction would provide insight into the mechanism of arfinactivation and its role in cell cycle control. A strain of C. albicans containing a null allele of its G protein α subunit gene, GPA1 (7). Genetic studies have shown that Nmt is essential for viability. A strain of C. albicans was constructed in which one copy of its NMT gene was deleted. A Gly⁴⁷→Asp mutation was introduced into the remaining NMT allele (designated nmt447D). This amino acid substitution produces a marked reduction in the enzyme’s catalytic efficiency at 24 and 37°C, reflecting, in part, a reduction in its affinity for myristoyl-CoA (8). Unlike isogenic NMT/NMT or NMT/nmtΔ strains, nmtΔ/nmt447D cells require myristate for their growth in rich media at 24 and 37°C. Removing myristate results in cell death (8). This lethality correlates with levels of cellular protein N-myristoylation. Arf is completely N-myristoylated, while its N-myristoylation is reduced by the nmtΔ/nmt447D mutation. This suggests that examination of the Nmt-Arf interaction would provide insight into the mechanism of Arfinactivation and its role in cell cycle control.
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CoA, and Pseudomonas acyl-CoA synthetase (12). The final reaction mixture (110 μl) contained variable amounts of peptide, 0.23 or 0.3 μM [3H]myristoyl-CoA, and purified C. albicans Nmt (10–100 ng). After a 10-min incubation at 24°C, the [3H]myristoylpeptide product was purified by reverse phase HPLC (12) and quantitated using an in-line flow scintillation detector (Radiomatic A250, Packard). K_m and V_max were calculated using nonlinear regression analysis of the initial velocities with the program lncat (version 1.55, Biometalics, Princeton, NJ). All experiments were performed in triplicate and assays were repeated on at least two separate occasions.

RESULTS AND DISCUSSION

Selection of a Parental Peptide to Define Elements Required for Recognition by C. albicans Nmt—Arf proteins are produced in a wide variety of species and many are known to be substrates for Nmts in vivo (e.g. Refs. 20–24). An octapeptide representing the N terminus of C. albicans Arf (GLTISKLFL-NH_2) is a substrate for purified C. albicans Nmt in vitro (K_m = 0.6 μM; V_max = 48,000 pmol/min/mg enzyme). GLYASKLF-NH_2 representing the N terminus of S. cerevisiae Arp2 is also accommodated by C. albicans Nmt (K_m = 0.4 μM). Previous studies had shown that a derivative of GLYASKLF-NH_2 containing a Phe^8 → Ser substitution (GLYASKLS-NH_2) is a high affinity substrate for S. cerevisiae Nmt1 (K_m = 0.07 μM; Ref. 12). Subsequent replacement of its Gly^1 with Ala (GLYASKLF-NH_2) yielded the first known high affinity competitive peptide inhibitor of an Nmt (K_i = 5 μM with purified S. cerevisiae Nmt1p). Based on these observations, we chose GLYASKLS-NH_2 to begin our identification of functional groups required by the binary C. albicans Nmt-seryl-CoA complex for recognition of its peptide ligands.

Scanning Alanine Mutagenesis of GLYASKLS-NH_2 Reveals the Importance of Residues 1, 5, and 6 in Recognition—Table I shows the kinetic effects of replacing residues in GLYASKLS-NH_2 with Ala. Substitution of Ala at position 1 (GLYALKLS-NH_2) represents addition of a methyl group to the α-carbon of Gly. The result is to transform a substrate to an inhibitor (IC_50 = 29 ± 4 μM; see Table I). Double-reciprocal plots established that the inhibition was competitive versus peptide (K_i = 15.3 ± 6.4 μM) and noncompetitive versus myristoyl-CoA (K_i = 31.2 ± 0.7 μM) (Fig. 1, A and B). GLYASKLS-NH_2 does not serve as a substrate: experiments employing a wide range of Nmt, [3H]myristoyl-CoA, and peptide concentrations failed to yield detectable amounts of product ([3H]myristoyl-ALYSKLS; see legend to Table I).

Substitution of an Ala for the Leu^8 in GLYASKLS-NH_2 replaces an isobutyl group with a methyl. There was a modest 3-fold reduction in K_m which was offset by a 4-fold drop in V_max. Substitution of an Ala for its Tyr^3 replaces a p-hydroxybenzyl
with a methyl and had insignificant effects on these kinetic parameters (Table I).

The importance of the α-methyl group of Ala4 was explored by placing Gly at this position (GLYGSKLS-NH2). Removal of the methyl group results in a 15-fold increase in $K_m$ and a 4-fold augmentation of $V_{max}$ (Table I).

Replacement of Ser5 with Ala can be viewed as a substitution of a hydrogen for a hydroxyl. The effect of this substitution is much more dramatic than any of the alterations at positions 1–4: $K_m$ increases by at least 10,000-fold and $V_{max}$ decreases 6-fold, resulting in a catalytic efficiency ($V_{max}/K_m$) which is 66,000-fold lower than that of the parent octapeptide, GLYASKLS-NH2 (Table I). The aminomethyl side chain in the adjacent Lys6 also appears to play an important role in recognition: replacement of this Lys with Ala results in a 236-fold increase in $K_m$ (Table I).

As in the case of Leu2, replacement of Leu7 with Ala produces only minor (2–3-fold) effects on $K_m$ and $V_{max}$ (Table I). Finally, unlike the dependence on the hydroxyl of Ser at position 5, replacement of Ser with Ala at position 8 produces no significant change in $K_m$ and a less than 2-fold alteration in $V_{max}$.

The results of the scanning alanine mutagenesis suggest that C. albicans Nmt recognizes the Arf substrate based in large part on the nature of functional groups present at residues 1, 5, and 6. This hypothesis was supported by an alanine scan of ALYASKLS-NH2 (Table I). Substitution of Leu2 increases inhibitory potency by 7-fold while placing Ala at positions 3 and 8 produces no significant change in IC50. Introducing Ala at positions 4 and 7 results in ~10-fold reductions in inhibitory activity. However, replacement of Ser5 and Lys6 changes the IC50 from 29 ± 4 to 1520 ± 50 μM and 2680 ± 50 μM, respectively (50- and 90-fold increases; Table I).

C-terminal truncations of the parental substrate (GLYASKLS-NH2) and inhibitor (ALYASKLS-NH2) also revealed the important contribution of Lys6. Comparison of GLYASKL-NH2, GLYASKNH2, and GLYAS-NH2 (Table I) established that (i) deletion of Ser5 has no significant effect on kinetic parameters; (ii) deletion of Leu2-Ser6 produces a modest 7-fold increase in $K_m$ and a 5-fold augmentation of $V_{max}$, and (iii) removal of Lys4-Leu5-Ser6 results in barely detectable amounts of product, even when enzyme and peptide concentrations are increased 10–100-fold over that used for assaying the other peptides. Similarly, loss of Leu2-Ser6 from ALYASKLS-NH2 only produces a 10-fold reduction in IC50 (Table I).

Further Characterization of Structure-Activity Relationships at Positions 1, 5, and 6 of ALYASKLS-NH2—We reasoned that
if positions 1, 5, and 6 of ALYASKLS-NH₂ provide essential elements for recognition by Nmt, then it might be possible to de-peptidize this parental inhibitor by removing the nonessential residues and replacing them with hydrocarbon linkers. However, before attempting such an exercise, we further defined structure-activity relationships at these three positions to obtain additional information about the spectrum of functional groups that might be incorporated into de-peptidized inhibitors.

Options for replacing the N-terminal amino acid were explored by examining the effects of (i) adding different α carbon substituents; (ii) removing, substituting for, or masking the nitrogen acceptor; and (iii) altering the distance between this nitrogen and the Ser-Lys dipeptide. The results are presented in Table II. A comparison of L- and D-Ala revealed that substitution of the D-isomer abolishes inhibitory activity (IC₅₀ = 29 ± 4 nM). This finding suggests that either an α-amino or methyl group, or both, in the R-configuration prevents binding to the binary myristoyl-CoA Nmt complex. Extension of the side chain at position 1 by substituting the methyl with either a propargyl or a propylguanidino group yielded compounds (S-propargylglycine-LYASKLS-NH₂ and N-α-arginyly-LYASKLS-NH₂) that have similar inhibitory activities (IC₅₀ = 51 ± 4 and 54 ± 6 μM, respectively) as L-alanyl-LYASKLS-NH₂. These results indicate that the peptide recognition site of the myristoyl-CoA Nmt complex is able to accommodate marked variations in the polarity and steric bulk, but not in the stereochemistry, of the α carbon side chain of residue 1.

When the primary amine of GLYASKLS-NH₂ is replaced with a hydroxyl (N-hydroxyacetyl-LYASKLS-NH₂), capped with an N-methyl group (N-sarcosyl-LYASKLS-NH₂), or its charge neutralized by acetylation (N-acetylglycyl-LYASKLS-NH₂), there are no remarkable effects on inhibitory activity (IC₅₀ changes <3-fold; Table II). When the amine nitrogen is removed entirely (N-acetyl-LYASKLS-NH₂), the IC₅₀ increases 8-fold (225 ± 2 μM). The hydroxyl, N-methyl, and N-acetylglycyl substituents all have heteroatom H-bond donor and acceptor groups that may mimic the amine present in GLYASKSL-NH₂, but which is absent from N-acetyl-LYASKLS-NH₂.

The distance between the terminal H-bond donor or acceptor group and the interior Ser-Lys dipeptide is sensed by the enzyme. N-Acetyl-LYASKLS-NH₂ can be viewed as an analog lacking a terminal amine but having a heteroatom more proximal to the Ser-Lys dipeptide than ALYASKLS-NH₂. In addition, altering the distance by substituting Ala with N-β-alanyl (IC₅₀ = 108 ± 1 μM) or N-methylcarbamoyl (IC₅₀ = 82 ± 3 μM) also compromises inhibitory activity (3–4-fold compared with their ALYASKLS-NH₂ parent).

Because of the potential to build product-like or transition state-like binding modes into a de-peptidized derivative, we also added two groups that extend from the terminal nitrogen: myristoyl (yielding the product N-myristoylglycyl-LYASKLS-NH₂) and an N-2,2'-difluoro-3-ketoheaxadecanoyl group (yielding a possible tetrahedral transition-state mimic; cf. Ref. 26). These compounds are only marginally better inhibitors (IC₅₀ = 5 ± 1 and 17 ± 0.3 μM, respectively) than ALYASKLS-NH₂.
Inhibitory potency of derivatives of ALYASKLS-NH$_2$ with substitutions at positions 5 and 6

| Amino acids | IC$_{50}$ ($\mu$M) | Amino acids | IC$_{50}$ ($\mu$M) |
|-------------|-------------------|-------------|-------------------|
| d-Ser       | 920 ± 110         | d-Lys       | 220 ± 30          |
| Ser(Omc)    | 522 ± 30          | Arg         | 105 ± 20          |
| Thr         | 500               | Orn         | 244 ± 5           |
| Asp         | >1000             | Nle         | 300               |

(29 ± 4 $\mu$M) (Table II).

Changing the stereochemistry of Ser$^5$ or O-methylation markedly attenuates the inhibitory activity of ALYASKLS-NH$_2$ (IC$_{50}$ increases to 920 ± 110 and 522 ± 30 $\mu$M; Table III). Neither the isosteric hydroxyl in Thr nor the OH group of Asp effectively substitutes for the side chain hydroxyl in l-Ser (Table III).

Substituting d-Lys at position 6 of ALYASKLS-NH$_2$ is deleterious (8-fold increase in IC$_{50}$ to 220 ± 30 $\mu$M) but is not as damaging as changing the stereochemistry at Ser$^5$ (30-fold increase; Table III). Arg$^6$ is inferior to Lys$^6$ (4-fold increase in IC$_{50}$) but is slightly better tolerated than the shortened aminoalkyl side chain of Orn (8-fold increase). Comparison of Nle$^6$ (10-fold increase in IC$_{50}$) and Lys$^6$ emphasizes the importance of a primary amine at this position (Table III).

De-peptidization of ALYASKLS-NH$_2$—The alanine scan of ALYASKLS-NH$_2$ established that amino acids 2 or 3 could be substituted with modest or no effects on inhibitory potency. As a prelude to de-peptidization, both Leu$^2$ and Tyr$^3$ of ALYASKLS-NH$_2$ were replaced by Ala (AAAASKLS-NH$_2$). This “simplified” compound was 7-fold more potent an inhibitor (IC$_{50}$ = 4 ± 1 $\mu$M) than its parent (Table IV).

Our initial approach for de-peptidization was to replace the N-terminal two residues of ALYASKLS-NH$_2$ with an aminoalcohol moiety of comparable length. Remarkably, when aminopenoyl was used to replace the 6 backbone atoms of Ala$^1$-Leu$^2$, the resulting compound, 5-aminopenoyl-YASKLS-NH$_2$, was 180-fold more potent than the starting octapeptide (IC$_{50}$ = 0.16 ± 0.01 versus 29 ± 4 $\mu$M; Table IV). Further kinetic analysis confirmed that it was a competitive inhibitor for peptide (K$_m$ = 0.133 ± 0.019 $\mu$M). Experiments employing a large (2,000-fold) excess of Nmt over that used in standard assays, a 10-fold increase in incubation time, and up to 1000 $\mu$M 5-aminopenoyl-YASKLS-NH$_2$ failed to yield detectable amounts of [H]$^3$myristoyl-peptide analog, thereby establishing that this compound does not serve as a Nmt substrate.

There were only minimal (3-fold) increases in IC$_{50}$ when the chain length of the aminoaalkyl group was adjusted by adding or subtracting one methylene (4-aminobutanoyl- and 6-aminohexanoyl-YASKLS-NH$_2$, respectively; Table IV). This indicates that (i) the fungal enzyme is able to both measure and tolerate one atom variation in the length of the flexible alkyl chain, and (ii) a 6-atom linker is the optimal length for replacement of the N-terminal depeptide.

As noted above, deletion of Ser$^5$ in ALYASKLS-NH$_2$ produces no effect on its inhibitory activity while deletion of Leu$^2$-Ser$^3$ results in 10-fold reduction in potency (cf. Table I). Virtually identical results were noted when these residues were deleted from 5-aminopenoyl-YASKLS-NH$_2$ (Table IV). Further C-terminal truncation, i.e. removing Lys from 5-aminopenoyl-YASKLS-NH$_2$, produced an inactive tripeptide (IC$_{50}$ >1000 $\mu$M) with only two of the three postulated essential elements for recognition.

When the o-methyl group of Ala$^4$ was removed in ALYASKLS-NH$_2$ and GLYASKLS-NH$_2$ by Gly substitution, the IC$_{50}$ increased 10-fold and the $K_m$ rose 15-fold, respectively.

Depeptidization of ALYASKLS-NH$_2$

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| 4-Aminobutanoyl-YASKLS-NH$_2$ | 0.53 ± 0.04 |
| 5-Aminopenoyl-YASKLS-NH$_2$ | 0.16 ± 0.01 |
| 6-Aminohexanoyl-YASKLS-NH$_2$ | 0.44 ± 0.03 |
| 11-Aminoundecanoyl-SKLS-NH$_2$ | 0.49 ± 0.04 |

Removal of the primary amine

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| Undecanoyl-SKLS-NH$_2$ | 12.7 ± 1.3 |
| Dodecanoyl-SKLS-NH$_2$ | 16.6 ± 1.3 |

Role of amide bonds

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| Glycyl-8-aminooctoyl-SKLS-NH$_2$ | 11.3 ± 1.2 |
| 5-Aminopenoyl-5-aminopenoyl-SKLS-NH$_2$ | 2.2 ± 0.3 |

C-terminal truncations

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| 5-Aminopenoyl-YASK..-NH$_2$ | 0.42 ± 0.01 |
| 5-Aminopenoyl-YASK...-NH$_2$ | 7.1 ± 0.14 |
| 5-Aminopenoyl-YASK...-NH$_2$ | >1000 |
| 11-Aminoundecanoyl-SK...-NH$_2$ | 1.2 ± 0.1 |
| 11-Aminoundecanoyl-SK...-NH$_2$ | 14.5 ± 1.6 |

Position 4 structure-activity series

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| 5-Aminopenoyl-YSKLS-NH$_2$ | 32 ± 4 |
| 5-Aminopenoyl-Y-d-alanoyl-SKLS-NH$_2$ | >1000 |
| 5-Aminopenoyl-Y-sarcosyl-SKLS-NH$_2$ | 7.2 ± 1.4 |
| 5-Aminopenoyl-Y-skls-NH$_2$ | 39 ± 1 |
| 5-Aminopenoyl-Y-VSKL-NH$_2$ | 0.17 ± 0.02 |
| 5-Aminopenoyl-YISKL-NH$_2$ | 0.40 ± 0.06 |
| 5-Aminopenoyl-YLSKL-NH$_2$ | 0.88 ± 0.32 |
| 5-Aminopenoyl-Y-phenylglycyl-SKLS-NH$_2$ | 61 ± 2 |
| 5-Aminopenoyl-Y-t-butylylanyl-SKLS-NH$_2$ | 25 ± 2 |
| 5-Aminopenoyl-YFSKL-NH$_2$ | 4.6 ± 0.1 |
| 5-Aminopenoyl-Y-cyclohexylalanyl-SKLS-NH$_2$ | 55 ± 2 |
| 5-Aminopenoyl-Y-norleucyl-SKLS-NH$_2$ | 2.6 ± 0.6 |
| 5-Aminopenoyl-Y-norvalyl-SKLS-NH$_2$ | 0.34 ± 0.07 |
| 5-Aminopenoyl-Y-propargylglycyl-SKLS-NH$_2$ | 1.25 ± 0.07 |
| 5-Aminopenoyl-Y-aminobutyryl-SKLS-NH$_2$ | 45 ± 0.3 |
| 5-Aminopenoyl-Y-cyclopentylglycyl-SKLS-NH$_2$ | 100 |

Position 5 structure-activity series

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| 11-Aminoundecanoyl-homeseryl-K-NH$_2$ | 170 |
| 11-Aminoundecanoyl-(4-hydroxypropyl)-K-NH$_2$ | 1000 |
| 11-Aminoundecanoyl-\(\text{K-NH}_2\) | 43 ± 4 |

Position 6 structure-activity series

| Peptide | IC$_{50}$ ($\mu$M) |
|---------|-------------------|
| 11-Aminoundecanoyl-S-d-lysyl-NH$_2$ | >1000 |
| 11-Aminoundecanoyl-S-norleucyl-L-NH$_2$ | 60 ± 2 |
| 11-Aminoundecanoyl-S-3-acetyllysyl-L-NH$_2$ | 98 ± 1 |
| 11-Aminoundecanoyl-SH-NH$_2$ | 21 ± 0.9 |
| 11-Aminoundecanoyl-S-d-histidyl-NH$_2$ | >1000 |
| 11-Aminoundecanoyl-S-1-methylhistidyl-NH$_2$ | 300 |
| 11-Aminoundecanoyl-S-3-methylhistidyl-NH$_2$ | >1000 |
| 11-Aminoundecanoyl-S-3-aminophenylalan-NH$_2$ | >1000 |

Therefore, before proceeding with further de-peptidization, we explored the importance of Ala in the minimal fully active, 5-aminopenoyl-containing compound (5-aminopenoyl-YASKLS-NH$_2$). Sixteen derivatives were examined (Table IV). The methyl group of Ala “remains” important: replacement with Gly results in a 76-fold reduction in inhibitory activity (IC$_{50}$ = 32 ± 4 versus 0.42 $\mu$M). The stereochemistry of this methyl is critical since substitution of l-Ala with d-Ala produces a >2000-fold reduction in potency. Moving the methyl group from the $\alpha$-carbon to the amide nitrogen (alanine →...
sarcosine) is poorly tolerated (17-fold reduction). Appending a hydroxyl to the methyl group (Ser) produces a 90-fold reduction in potency. Amino acids with branched, moderately bulky side chains (Val, Ile, and Leu) are equivalent to or slightly better than Ala (IC\textsubscript{50} = 0.17–0.88 \mu M). Substituents with greater bulk, phenylglycine, \textit{t}-butylalanine, phenylalanine, cyclohexylalanine, and norleucine, are deleterious (IC\textsubscript{50} values increase 6–145-fold relative to 5-aminopentanoyl-YASKLNH\textsubscript{2}). Norvaline and propargylglycine contain slightly smaller unbranched side chains that are tolerated by Nmt (IC\textsubscript{50} = 0.34 ± 0.17 and 1.25 ± 0.07 \mu M, respectively). The geminal di-substituted aminoisobutyrate and cyclopropylglycine residues both have a pronounced negative impact on inhibition (IC\textsubscript{50} = 730–732 \mu M). Again stereochemistry is important: \textit{d}-His is inactive (IC\textsubscript{50} > 1000 \mu M). Masking either of the imidazole nitrogens (1-methyl-histidine or 3-methyl-histidine) also eliminates inhibitory activity (IC\textsubscript{50} > 1000 \mu M). p-Aminophenylalanine contains a terminal basic amine: the length of the side chain is similar to Lys, although the bulk is greater and the pK\textsubscript{a} of the amine is lower (−10 versus −5). It cannot substitute for Lys (IC\textsubscript{50} > 1000 \mu M; Table IV).

**Prospectus**—Our findings indicate that an 11-aminoundecanoyl backbone can serve to maintain an appropriate distance between three elements critical for recognition by the peptide-binding site in the fungal Nmt-myristoyl-CoA binary complex. These elements include a simple \omega-terminal amino group, a \beta-hydroxyl, and an \epsilon-amino group or an imidazole. Each of these competitive inhibitors has one peptide bond and two chiral centers. They are equipotent with the starting peptide inhibitor, ALYASKLNH\textsubscript{2}, which was derived from a known Nmt substrate (Arg) and contained 7 peptide bonds and 8 chiral centers. 11-Aminoundecanoyl-SKLNH\textsubscript{2} and 11-amino-undecanoyl-SKLNH\textsubscript{2} exhibit a competitive pattern of inhibition (K\textsubscript{i} = 0.40 ± 0.03 \mu M).

**Structure-Activity Studies of 11-Aminoundecanoyl-SKLS-NH\textsubscript{2}**—This synthetic organic-peptide hybrid retains the three critical elements of recognition defined from the original alanine scan and truncations of ALYASKLNH\textsubscript{2}: an N-terminal primary amine, a Ser\textsuperscript{\epsilon}-hydroxyl, and an \epsilon-amino group at position 6. Removing the primary amine from 11-aminoundecanoyl-SKLNH\textsubscript{2} (undecanoyl-SKLNH\textsubscript{2}), or replacing it with a methyl group (dodecanoyl-SKLNH\textsubscript{2}), results in substantial reductions in inhibitory potency (26- and 34-fold, respectively, as defined by IC\textsubscript{50}; Table IV), thereby confirming the importance of this amine for recognition by Nmt.

**Substitution of the N-terminal tetrapeptide of ALYASKLNH\textsubscript{2} with an 11-aminoundecanoyl group removes three backbone amide bonds.** The contribution of these bonds to recognition was evaluated by preparing two compounds, one with an amide bond analogous to that linking Ala\textsuperscript{1}-Leu\textsuperscript{2} (glycyl-8-aminooctanoyl-SKLNH\textsubscript{2}), the other with a bond analogous to that linking Leu\textsuperscript{2}-Tyr\textsuperscript{3} (5-aminopentanoyl-5-aminopentanoyl-SKLS-NH\textsubscript{2}). In both cases, introduction of the amide bonds diminished inhibitory potency relative to 11-aminoundecanoyl-SKLNH\textsubscript{2} (23- and 5-fold respectively; Table IV). These results further emphasize the surprising nature of the finding that this peptide N-myristoyltransferase seems to “prefer” competitive peptidomimetic inhibitors that lack peptide bonds.

**11-Aminoundecanoyl-SK-NH\textsubscript{2}.** A Dipetide Inhibitor that Retains Critical Elements of Recognition—As with ALYASKLNH\textsubscript{2} and 5-aminopentanoyl-YASKLS-NH\textsubscript{2}, deletion of the C-terminal Ser from 11-aminoundecanoyl-SKLNH\textsubscript{2} produces only a minimal (3-fold) effect on its inhibitory activity while deletion of its C-terminal Leu-Ser dipeptide (11-amino-undecanoyl-SK-NH\textsubscript{2}) results in a more substantial (30-fold) reduction in potency. 11-Aminoundecanoyl-SK-NH\textsubscript{2} represents a dipetide inhibitor (IC\textsubscript{50} = 14.5 ± 1.6 \mu M) that is competitive for peptide (K\textsubscript{i} = 11.7 ± 0.4 \mu M; Fig. 1C). The K\textsubscript{i} of this compound is equivalent to the K\textsubscript{i} of the starting octapeptide inhibitor, ALYASKLNH\textsubscript{2} (15.3 ± 6.4 \mu M).

The contributions of the remaining amino acids in 11-amoundecanoyl-SK-NH\textsubscript{2} to recognition were explored. Presentation of the hydroxyl group is important: extension of this OH in the context of homoserine, cis-4-hydroxyproline, or tyrosine worsens inhibitory potency by 3–70-fold (Table IV). The stereochemistry of the Lys side chain is also critical: substituting \textit{p}-Lys abolishes inhibitory activity (Table IV). Deletion of the \epsilon-amino group (Nle) or masking its charge (\epsilon-N-acetyl-lysine) produces more modest attenuation (4–7-fold). In the context of this dipetide aminoalkyl inhibitor, 1-His can substitute for \textit{p}-Lys, producing an inhibitor competitive for peptide with similar potency (K\textsubscript{i} = 11.9 ± 1.0 versus 11.7 ± 0.4 \mu M). Again stereochemistry is important: \textit{d}-His is inactive (IC\textsubscript{50} > 1000 \mu M). Masking either of the imidazole nitrogens (1-methyl-histidine or 3-methyl-histidine) also eliminates inhibitory activity (IC\textsubscript{50} > 1000 \mu M). p-Aminophenylalanine contains a terminal basic amine: the length of the side chain is similar to Lys, although the bulk is greater and the pK\textsubscript{a} of the amine is lower (−10 versus −5). It cannot substitute for Lys (IC\textsubscript{50} > 1000 \mu M; Table IV).

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Scanning Alanine Mutagenesis and De-peptidization of a Candida albicans Myristoyl-CoA:Protein-N-Myristoyltransferase Octapeptide Substrate Reveals Three Elements Critical for Molecular Recognition

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