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The Isoprenoid Substrate Specificity of Isoprenylcysteine Carboxylmethyltransferase

DEVELOPMENT OF NOVEL INHIBITORS*

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Isoprenylcysteine carboxylmethyltransferase (Icmt) is an integral membrane protein localized to the endoplasmic reticulum of eukaryotic cells that catalyzes the post-translational α-carboxylmethylesterification of C*AAX* motif proteins, including the oncoprotein Ras. Prior to methylation, these protein substrates all contain an isoprenylcysteine residue at the C terminus. In this study, we developed a variety of substrates and inhibitors of Icmt that vary in the isoprene moiety in order to gain information about the nature of the lipophilic substrate binding site. These isoprenoid-modified analogs of the minimal Icmt substrate *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) were synthesized from newly and previously prepared farnesol analogs. Using both yeast and human Icmt enzymes, these compounds were found to vary widely in their ability to act as substrates, supporting the isoprenoid moiety as a key substrate recognition element for Icmt. Compound 3 is a competitive inhibitor of overexpressed yeast Icmt (K_I = 17.1 ± 1.7 μM). Compound 4 shows a mix of competitive and uncompetitive inhibition for both the yeast and the human Icmt proteins (yeast K_{IC} = 35.4 ± 3.4 μM, K_{IU} = 614.4 ± 148 μM; human K_{IC} = 119.3 ± 18.1 μM, K_{IU} = 377.2 ± 42.5 μM). These data further suggest that differences in substrate specificity exist between the human and yeast enzymes. Biological studies suggest that inhibition of Icmt results in Ras mislocalization and loss of cellular transformation ability, making Icmt an attractive and novel anticancer target. Further elaboration of the lead compounds synthesized and assayed here may lead to clinically useful higher potency inhibitors.

Ras proteins, notably wild-type and oncogenic K-Ras as well as many other important signal transduction proteins, must undergo post-translational modification to function in eukaryotic cells (1–3). These proteins contain a signature C-terminal C*AAX* box motif, where C is cysteine, *A* is generally an aliphatic residue, and *X* can be one of several different amino acids (Fig. 1*A*). This consensus sequence is recognized by one of two isoprenyltransferases, FTase[1] (protein-farnesyltransferase) or GGTase I (protein-geranylgeranyltransferase I), which transfers either a 15- or 20-carbon isoprene moiety, respectively, to the cysteine residue. Ras and certain other proteins are farnesylated, but the majority of naturally occuring C*AAX* proteins are geranylgeranylated. Subsequent to isoprenylation, the three -*AAX* residues are removed by the endoproteases Rce1 or Ste24, (4–6), and the resulting cysteine carboxylate is methylated by the *S*-adenosylmethionine-dependent isoprenylcysteine carboxylmethyltransferase, Icmt (7–12). The overall effect of this trio of post-translational modification steps is an increase in the hydrophobicity of the modified protein, directing otherwise soluble proteins to their proper intracellular membrane location.

Ste14p from *Saccharomyces cerevisiae* is the founding member of a homologous family of Icmt enzymes present in all eukaryotic organisms (13). Ste14p is a 26-kDa integral membrane protein localized to the endoplasmic reticulum membrane and contains six putative transmembrane spans. A 33-kDa functional human ortholog of the yeast protein, hIcmt (also called pcCMT), which is also localized to the endoplasmic reticulum membrane, was recently identified and characterized (11). The yeast and human Icmt share 41% identity and 63% similarity, overall, suggesting that their three-dimensional structures and mechanisms of action are similar. In fact, human Icmt expressed in yeast complements a *ste14*Δ deletion (11). We have recently overexpressed both yeast and human Icmt in *S. cerevisiae* to high levels and characterized their activities *in vitro* (14, this study). Both of these Icmt variants recognize and modify both farnesylated and geranylgeranylated substrates (1, 2, 12, 14) and are evaluated in the present studies to compare their substrate preferences.

To date, the nature of the isoprenylcysteine binding site in all Icmt enzymes remains relatively unexplored. Previous studies have indicated that the isoprene moiety is a crucial recognition element for Icmt, as the minimal compounds *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC) are good substrates (1, 2, 12, 14, 15) and are recognized equivalently by the enzyme (14). Furthermore, aminoacyl modifications were also shown to block protein methylation by crude

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[1] The abbreviations used are: FTase, protein farnesyltransferase; GGTaseI, protein geranylgeranyltransferase I; Rce1, Ras-converting enzyme 1; Icmt, isoprenylcysteine carboxylmethyltransferase; FPP, farnesylpyrophosphate; AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S*-geranyl-L-cysteine; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; ESI, electrospray ionization.

Icmt (16, 17). In this study, to explore the substrate specificity of Icmt, we focused on the isoprene moiety itself via the synthesis of *N*-acetyl isoprenylcysteine analogs. Our goals are to use these synthetic compounds as tools for further biochemical exploration of the mechanism of action of the enzyme and starting points for the development of inhibitors.

Icmt has emerged as a particularly intriguing target for potential anticancer agents, especially against K-Ras. K-Ras is the most commonly mutated form of Ras found in human malignancies, particularly in solid malignancies (18). Targeted inactivation of the *Icmt* gene in mammalian cells led to a profound mislocalization of K-Ras and a blockage of its ability to promote cellular transformation (19–21). It has also been demonstrated that blocking the methylation of K-Ras blocks its association with microtubules, which may be crucial for the localization and biological activity of this Ras variant. These findings suggest that the development of inhibitors of the methylation step may prove to be useful for the treatment of human cancers (22, 23).

Recently, potent FTase inhibitors have exhibited promise as anticancer agents in human clinical trials. These inhibitors are thought to have multiple cellular targets, including some Ras proteins. Although these agents can inhibit the growth of H-Ras tumors, they have surprisingly little effect on many K-Ras-transformed tumor types. Importantly, K-Ras can be alternatively geranylgeranylated in the presence of FTase inhibitors whereas H-Ras is not (24, 25). This alternative modification by GGTase I (24, 25) apparently allows mutant K-Ras to continue its growth-promoting actions. Recent studies with Icmt$^{-/-}$ fibroblasts have indicated that the methylation of K-Ras proteins by Icmt plays a central role in the cellular localization and transformation ability of this key oncoprotein (19–21). Combined, these data provide compelling reasons that inhibitors of Icmt have great potential as novel anticancer agents (22, 23).

Many signaling proteins undergo -C*AAX* processing, and inhibiting Icmt could also target the abnormally high activity of these other signaling proteins in tumor cells, regardless of the specific prenyl group attached to the protein. However, although a recent study confirms that methylation is required for the proper localization of Ras, it also demonstrates that the modification is not necessary for localization of the Rho proteins, another class of C*AAX* proteins (26). This differential effect was linked to the fact that Ras is farnesylated, and the Rho proteins are geranylgeranylated, suggesting that Icmt inhibition will have a much more profound inhibitory effect on the activity of farnesylated proteins, such as Ras, than geranylgeranylated proteins.

To study both the nature of the substrate binding site as well as to develop potentially useful inhibitors of Icmt, modified analogs of the minimal Icmt substrate AFC, **1** (Fig. 1*B*), were synthesized (Fig. 2) (27) and evaluated as substrates and inhibitors of Icmt using crude membrane preparations from yeast cells overexpressing either Ste14p or human Icmt. These compounds were found to vary widely in their ability to act as substrates, and revealed structural requirements of the key isoprene moiety necessary for recognition by Icmt. Two of the compounds synthesized, the isobutenyl derivative, **3**, and the isobutenyl biphenyl derivative, **4**, were selected for further study because of their minimal substrate activity, combined with significant inhibitory activity. Both compounds were found to be inhibitors of both yeast and human Icmt in micromolar concentrations. Such inhibitors may be valuable lead compounds for the development of novel anticancer agents (22, 23).

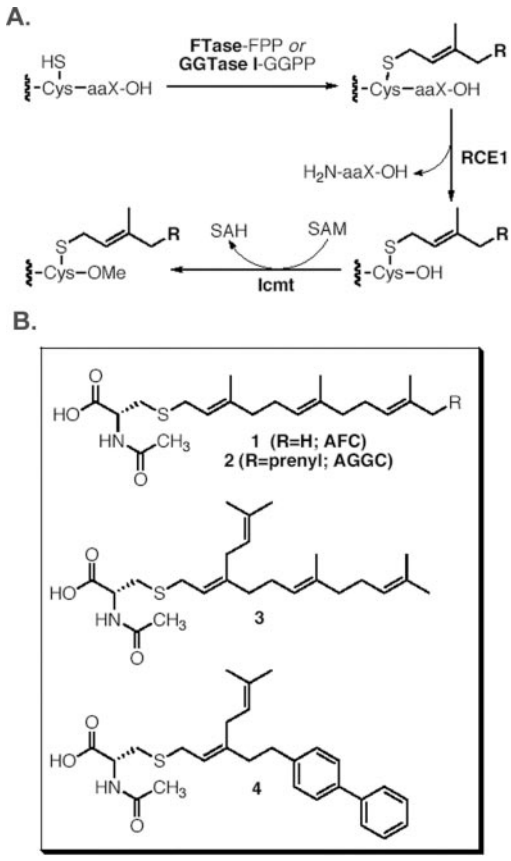

FIG. 1. *A*, C*AAX* protein processing in eukaryotes. *B*, isoprene-modified analogs of AFC (**1**). Compound **3**, an isobutenyl derivative of AFC (**1**). Compound **4**, an isobutenyl biphenyl derivative of AFC (**1**).

EXPERIMENTAL PROCEDURES

Synthesis of Analogs—The AFC analogs described here, compounds **3** and **4** (Fig. 1*B*), were synthesized from farnesol analogs that were prepared using close variants of our reported procedures (28–33). Detailed methods for the synthesis of **3** and **4** are given below and outlined in Fig. 2, *A* and *B*. Descriptions of the syntheses of the intermediates for the preparation of **4** are also given, along with proton NMR and MS characterization data.

N-Acetyl-S-(3-(3-methylbut-2-enyl)-7,11-dimethyldodeca-2Z,6E,10-trien-1-yl)-L-cysteine (Compound 3)—Chloride **8** (64 mg, 0.24 mmol, 1 equivalent; details for the synthesis of **8** are presented elsewhere) (33) and *N*-acetyl-L-cysteine (50 mg, 0.3 mmol, 2 equivalents) were dissolved in 7.0 N NH_3/MeOH (10 ml/mmol chloride), stirred at 0 °C for 1 h, and then at 20 °C for 1 h. The resulting mixture was concentrated by rotary evaporation. The crude compound was taken up in MeOH/CH_2Cl_2 and directly purified by flash column (gradient of 10–30% methanol in CH_2Cl_2) to afford compound **3** in a 60% yield of 61 mg, based on the alcohol **7.** ^{1}H NMR (300 MHz, $CDCl_3$): 1.57 (s, 6H), 1.63 (s, 3H), 1.70 (two s, 6H), 2.0–2.1 (narrow m, 14H), 2.71 (narrow m, 2H), 2.9 (br, 2H), 3.16 (narrow m, 2H), 4.7 (narrow m, 1H), 4.95 (app t, 1H), 5.11 (app t, 2H), 5.24 (t, 1H), 6.45 (d, 1H), 9.3–9.7 (very br, 1H). ^{13}C (75 MHz, $CDCl_3$) 16.42, 18.09, 18.3, 23.36, 26.11, 27.12, 30.49, 34.02, 37.42, 40.11, 122.38, 124.24, 124.74, 131.02, 132.67, 135.74, 144.307, 171.52. MS-ESI (M-H) = 420. Elemental Analysis: calculated for $C_{24}H_{38}NO_3SK_{0.70}Na_{0.30}$: C, 63.38; H, 8.42; found: C, 63.28, H, 8.52.

Ethyl 3-(But-3-methyl-2-en-1-yl)-5-(4-phenyl)phenylpent-2Z-enoate (10)—Triflate **9** (synthesized as previously described, Ref. 29; 350 mg, 0.78 mmol), CuO (620 mg, 7.8 mmol), Ph_3As (23 mg, 0.078 mmol), and bis(benzonitrile)-palladium (II) chloride (16.5 mg, 0.0429 mmol) were placed in an argon-flushed flask and dissolved in *N*-methylpyrrolidone (6 ml). The mixture was immersed in an oil bath maintained at a temperature of 100–104 °C, (3-methylbut-2-enyl)tributyltin (0.393 ml, 1.17 mmol) was added, and the reaction mixture was stirred for 12 h. It was then cooled, taken up in ethyl acetate (25 ml), and washed with aqueous KF (2 × 20 ml) and H_2O (2 × 20 ml). The aqueous layers were back-extracted with ethyl acetate (30 ml), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated. Purification by

flash chromatography (hexane/ethyl acetate 98:2) gave **10**, in an 83% yield (230 mg). ^{1}H NMR (300 MHz, $CDCl_3$): 1.3 (t, 3H), 1.8 (t, 6H), 2.5 (t, 2H), 2.9 (t, 2H), 3.6 (d, 2H), 4.3 (q, 2H), 5.3 (t, 1H), 5.8 (t, 1H), 7.2 (t, 2H), 7.3 (t, 1H), 7.35 (d, 2H), 7.45 (d, 2H), and 7.5 (d, 2H).

3-(But-3-methyl-2-en-1-yl)-5-(4-phenyl)phenylpent-2Z-en-1-ol (11)—Compound **10** (230 mg, 0.65 mmol) was dissolved in anhydrous toluene (3 ml) and chilled to −78 °C. A 1.0 M solution of DIBAL-H (1.83 ml, 1.83 mmol) was added dropwise. The solution was stirred for 1 h at −78 °C and was then warmed slightly and quenched with 10% aqueous sodium potassium tartrate. The layers were separated, and the aqueous layer was extracted (3 × 20 ml) with ethyl acetate. The organic layers were combined, washed with brine (10 ml), dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (hexane/ethyl acetate 90:10) gave **11**, in a 76% yield (150 mg). ^{1}H NMR (300 MHz, $CDCl_3$): 1.8 (t, 6H), 2.5 (t, 2H), 2.9 (t, 2H), 3.6 (d, 2H), 4.1 (d, 2H), 5.3 (t, 1H), 5.8 (t, 1H), 7.2 (t, 2H), 7.3 (t, 1H), 7.35 (d, 2H), 7.45 (d, 2H), and 7.5 (d, 2H).

1-Chloro-3-(but-3-methyl-2-en-1-yl)-5-(4-phenyl)phenylpent-2Z-ene (12)—NCS (*N*-chlorosuccinimide; 55 mg, 0.39 mmol) was dissolved in CH_2Cl_2 (distilled from CaH_2), and the resulting solution was cooled to −30 °C with a dry ice/acetonitrile bath. Dimethyl sulfide (0.028 ml, 0.39 mmol) was added dropwise by syringe, and the mixture was warmed to 0 °C, maintained at that temperature for 15 min, and cooled to −30 °C. To the resulting milky white suspension was added dropwise a solution of the alcohol **11** (80 mg, 0.26 mmol; dissolved in CH_2Cl_2). The suspension was warmed to 0 °C and stirred for 3 h. The ice bath was removed, and reaction mixture was warmed to room temperature and stirred for an additional 2 h. The resulting solution was washed with hexane (2 × 20 ml). The hexane layers were then washed with brine (2 × 20 ml) and dried over $MgSO_4$. The compound was further elaborated to compound **4** without any purification. ^{1}H NMR (300 MHz, $CDCl_3$): 1.8 (t, 6H), 2.5 (t, 2H), 2.9 (t, 2H), 3.6 (d, 2H), 4.0 (d, 2H), 5.3 (t, 1H), 5.8 (t, 1H), 7.2 (t, 2H), 7.3 (t, 1H), 7.35 (d, 2H), 7.45 (d, 2H), and 7.5 (d, 2H).

N-Acetyl-S-(3-(3-methylbut-2-enyl)-5-(4-phenyl)phenylpent-2Z-en-1-yl)-L-cysteine (Compound 4)—Chloride **12** (70 mg, 0.216 mmol, 1 equivalent) and *N*-acetyl-L-cysteine (39 mg, 0.238 mmol, 1.1 equivalents) were dissolved in 7.0 N NH_3/MeOH (10 ml/mmol chloride), stirred at 0 °C for 1 h and then at 20 °C for 1 h. The resulting mixture was concentrated by rotary evaporation. The crude compound was taken up in MeOH/CH_2Cl_2 and directly purified by flash column (eluting with a gradient of 10–30% methanol in CH_2Cl_2) to afford compound **4** in a 50% yield (49 mg), based on the alcohol **11.** ^{1}H NMR: (300 MHz, $CDCl_3$) 1.63 (s, 6H), 2.4 (t, 2H), 2.8 - 3.0 (m, 6H), 3.2 (m, 2H) 4.7 (m, 1H), 5.2 (t, 1H), 5.5 (t, 1H), 7.2 (t, 2H), 7.3 (t, 1H), 7.35 (d, 2H), 7.45 (d, 2H), and 7.5 (d, 2H). MS ESI (M-H) = 450.

Yeast Strains and Media—Plasmid-bearing strains were created by transformation of the indicated plasmid into SM1188, which does not express Ste14p, using the method of Elble (34) with the following modification: dithiothreitol was added to a final concentration of 50 mM to increase the transformation efficiency. All strains were grown at 30 °C on synthetic complete solid media without uracil (SC-URA). The SM1188 strain was kindly provided by S. Michaelis (Johns Hopkins University School of Medicine).

Cloning of His-Ste14p—pCHH10m3N-STE14 (His-Ste14p), which encodes Ste14p with a 10× histidine tag followed by a triply iterated *myc* epitope repeat at the N terminus under the constitutive control of the phosphoglycerate kinase (*PGK*) promoter, was constructed as previously described (14).

Cloning of Human Icmt—Wild-type human Icmt (hIcmt) cDNA was a generous gift from S. Michaelis (Johns Hopkins University School of Medicine). The *hIcmt* gene was amplified by PCR after removal of an internal EagI restriction site, which does not result in a change in the amino acid sequence, and the product cloned into the pCHH10m3N vector. The resulting plasmid was pCHH10m3N-hIcmt (His-hIcmt). The plasmid was sequenced bidirectionally to confirm the DNA sequence.

Isolation of Membrane Fraction from Yeast Cells—Membrane fractions from yeast cells were isolated as previously described (14). Membrane protein concentration was determined using Coomassie Plus Protein Assay Reagent (Pierce) according to the manufacturer's instructions, and compared with a bovine serum albumin standard curve prepared by the same procedure.

Immunoblot Analysis—Protein samples in the presence of 1× Laemmli sample buffer were heated to 65 °C for 15 min and resolved by 12% SDS-PAGE. Proteins were transferred to a pure nitrocellulose membrane (0.2 μm; Schleicher & Schuell BioScience GmbH) at 400 mA for 1 h. The filter was blocked with 20% milk in phosphate-buffered saline with Tween (PBST; 137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.05% Tween 20, pH 7.4) and then incubated with the primary antibody (1:10,000 α-*myc*) dissolved in 5% milk in PBST for 3 h at room temperature. Following three washes with PBST, the filter was incubated with the secondary antibody (1:4,000 goat α-mouse horseradish peroxidase). After several washes with PBST, protein bands were visualized by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce).

In Vitro Vapor Diffusion Methyltransferase Assay—Methyltransferase assays were performed as described (7, 9, 35, 36) with minor modifications. The assay mixture contained a total volume of 60 μl and a final Tris-HCl concentration of 100 mM, pH 7.5. All reactions contained 20 μM *S*-adenosyl-[^{14}C-*methyl*]methionine and 5 μg of membrane protein from the His-Ste14p-overexpressing strain CH2704 or the His-hIcmt-overexpressing strain CH2766. Substrate curves were generated by varying the amount of compound in each reaction. Inhibition curves were generated by varying the amount of compound in each reaction while in the presence of 33 μM AFC. K_I curves were generated by varying the amount of AFC in the presence of a constant concentration of **3** or **4**. **3** was dissolved in ethanol. **1** and **4** were dissolved in dimethylsulfoxide. In all cases, the solvent was kept at a final concentration of <1% (v/v). The 60-μl reactions were incubated at 30 °C for 30 min. The reaction was stopped with the addition of 50 μl of 1 M NaOH/1% SDS. 100 μl of this mixture was spotted on folded filter paper (5.5 cm × 1.5 cm) and lodged in the neck of a scintillation vial containing 10 ml of scintillation fluid and capped to allow for diffusion of the released [^{14}C]methanol into the scintillant. The filters were pulled out after 2–3 h, the vials were shaken, and counted in a liquid scintillation analyzer.

Substrate and Inhibition Assays with Purified His-Ste14p Protein—His-Ste14p was purified as previously described (14), except samples were eluted in buffer containing 200 mM EDTA, pH 8.0 instead of 1 M imidazole. For substrate curves with purified His-Ste14p, liposomes containing substrate were prepared by rapid filtration through a Sephacryl S-100 high resolution (Amersham Biosciences) column, the substrate concentration was quantified, and the liposomes used in *in vitro* vapor diffusion methyltransferase assays as previously described (14). For inhibition assays, both the substrate, AFC, and the inhibitor, either compound **3** or compound **4**, were incubated with lipid. The substrate was at a constant concentration (50 μM), and the inhibitor was in increasing concentrations. The purified protein was reconstituted, and *in vitro* vapor diffusion methyltransferase assays were performed as previously described (14). All methyltransferase assays using purified His-Ste14p contained 89 ng of protein, which gives an enzyme concentration in the reaction of 42 nM.

Data Analysis—Apparent K_m and V_{max} values were estimated by fitting substrate curves to the Michaelis-Menten equation (see Equation 1) using GraphPad Prism 4. For compounds **3** and **4**, inhibition data were plotted as the log of inhibitor concentration *versus* percent activity. These curves were then fit to a variable slope sigmoidal dose-response curve to determine the IC_{50} and its 95% confidence interval. For compound **3**, experimental data were globally fit to Equation 2 to determine K_I. For compound **4**, experimental data were globally fit to Equation 3 to determine K_{IU} and K_{IC}. The K_m and V_{max} were held constant across all four curves. The data in Fig. 8 are depicted in the form of a double reciprocal plot, for ready visualization of the competitive or mixed nature of the inhibition of Icmt activity by **3** and **4**.

$$V = \frac{V_{max}[S]}{(K_m + [S])} \quad \text{(Eq. 1)}$$

$$V = \frac{V_{max}[S]}{\left(K_m\left(1 + \frac{[I]}{K_I}\right) + [S]\right)} \quad \text{(Eq. 2)}$$

$$V = \frac{V_{max}[S]}{\left(K_m\left(1 + \frac{[I]}{K_{IC}}\right) + [S]\left(1 + \frac{[I]}{K_{IU}}\right)\right)} \quad \text{(Eq. 3)}$$

RESULTS

Synthesis of AFC Analogs—The AFC analogs **3** and **4**, synthesized from the corresponding farnesol analogs as described under "Experimental Procedures," are shown in Fig. 1*B*. The synthetic scheme for the preparation of **3** is illustrated in Fig. 2*A*. The previously described triflate **5** was coupled with tributyl(3-methyl-2-butenyl)tin using modified Stille coupling conditions (33, 37) to give the ester **6**. The

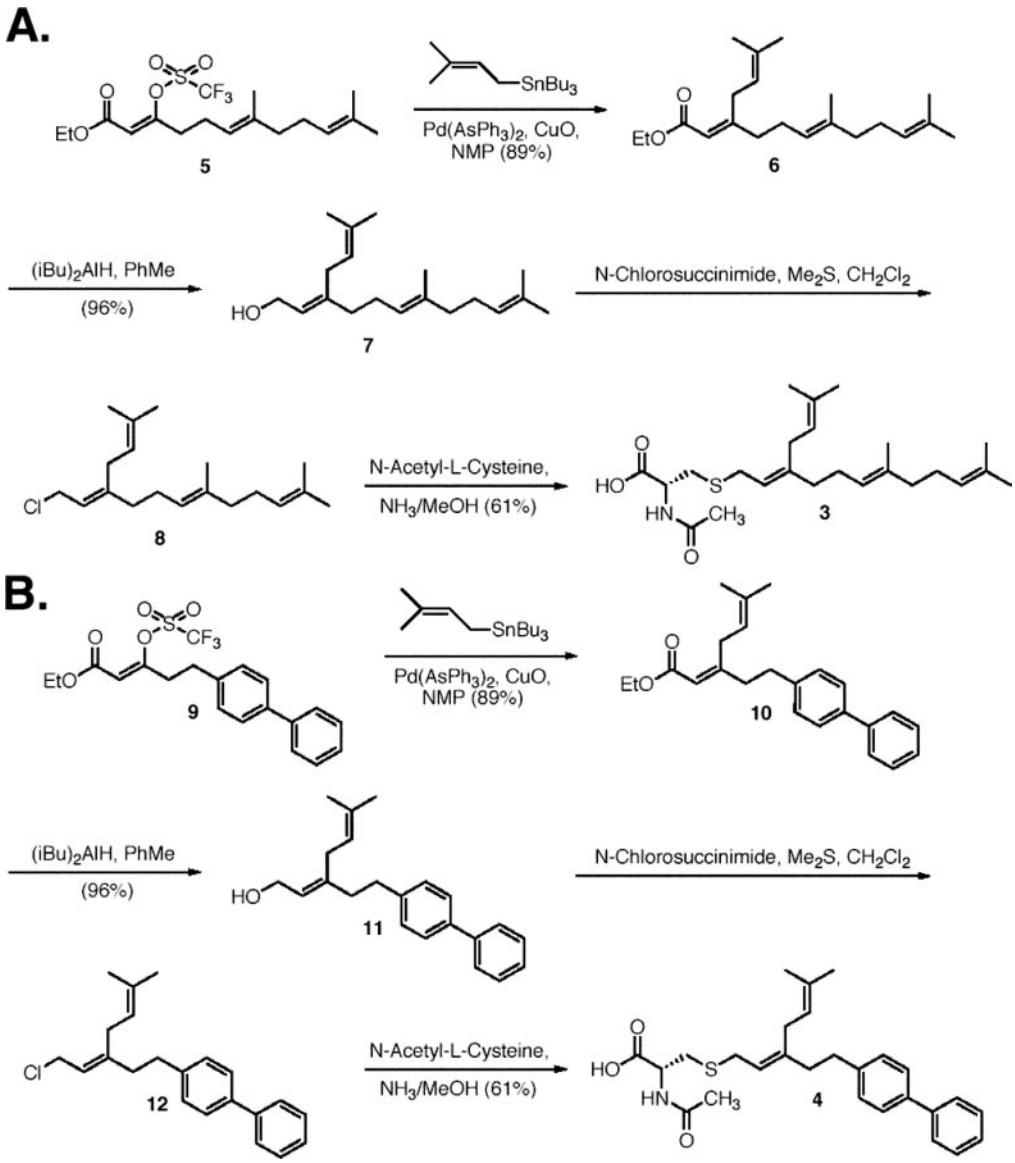

FIG. 2. *A*, synthesis of compound **3**. *B*, synthesis of compound **4**.

ester was reduced in excellent yield (96%) to afford alcohol **7**. The alcohol was then converted to the chloride **8** via the Corey-Kim chlorination reaction (38). The isoprenoid chloride **8** was coupled with *N*-acetyl-L-cysteine in ammonia/methanol, using a variant of the procedure originally described by Poulter and co-workers (27), to give the desired AFC analog **3**. The synthesis of the other promising inhibitor developed in this study, **4**, is illustrated in Fig. 2*B*. It utilizes the same synthetic route, but starts from the previously described biphenyl triflate **9** (29), as detailed under "Experimental Procedures."

Overexpression of His-Ste14p and His-hIcmt in S. cerevisiae—The *S. cerevisiae* Icmt, Ste14p, and the human Icmt (hIcmt) expressed in yeast were used as the model enzymes to screen the panel of potential Icmt substrates and inhibitors, with a goal of understanding the nature of the isoprene binding site, as well as the development of inhibitors. A plasmid was engineered coding for Ste14p that is N-terminally tagged with 10 histidine residues and a triple iteration of the *myc* epitope under the control of the constitutive phosphoglycerate kinase (*PGK*) promoter. This plasmid was expressed in the *S. cerevisiae* strain SM1188, a *STE14* deletion strain. The resulting strain (CH2704) expresses this tagged Ste14 variant, referred to simply as His-Ste14p. Human Icmt (hIcmt) was also cloned into the same parent vector, yielding His-hIcmt and was transformed into SM1188. The resulting strain (CH2766) expresses this tagged hIcmt variant, and the protein expressed is referred to as His-hIcmt. Crude membrane preparations containing either His-Ste14p or His-hIcmt were isolated for use in activity assays as described under "Experimental Procedures."

His-hIcmt is expressed at approximately half the level of His-Ste14p as determined by immunoblot analysis with an *α-myc* antibody (Fig. 3*A*). This expression level was corroborated by the *in vitro* vapor diffusion methyltransferase assay with AFC as the substrate. The specific activity of His-hIcmt from CH2766 membranes was approximately half that of CH2704 His-Ste14p membranes (Fig. 3*B*).

AFC Analogs as His-Ste14p and His-hIcmt Substrates—The ability of compounds **3** and **4** to act as either His-Ste14p or His-hIcmt substrates was assessed by performing the *in vitro* vapor diffusion methyltransferase assay with crude membranes, replacing AFC with increasing concentrations of each compound (Fig. 4 and Table I). 3-(isobutenyl)Farnesol (**7**), which lacks the cysteine portion of the molecule, and *S*-farnesylthioacetic acid are not His-Ste14p substrates, as measured

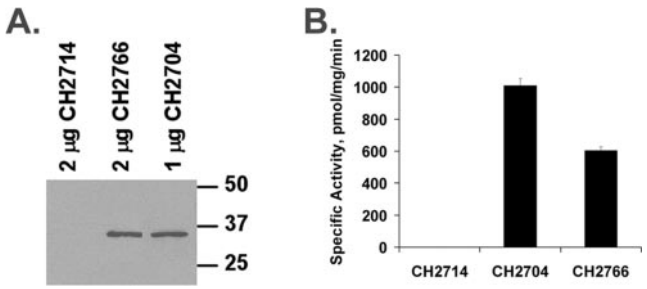

FIG. 3. *A*, expression level of CH2766 and CH2704. 1 μg of total crude membrane protein from CH2704 (His-Ste14p) and 2 μg of total crude membrane protein from the empty vector negative control CH2714 (Δ*ste14*) and CH2766 (His-hIcmt) crude were subjected to SDS-PAGE analysis, and the proteins were visualized by immunodetection with an *α-myc* monoclonal antibody (1:10,000). Protein bands were visualized by enhanced chemiluminescence following incubation with a horseradish peroxidase-conjugated secondary antibody (α-mouse 1:4,000). *B*, *in vitro* methyltransferase activity of His-Ste14p and His-hIcmt in crude membranes. 5 μg of total protein from crude membrane preparations were incubated with 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine and 200 μM AFC for 30 min at 30 °C and activity quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments done in duplicate. *Error bars* represent ± 1 S.E.

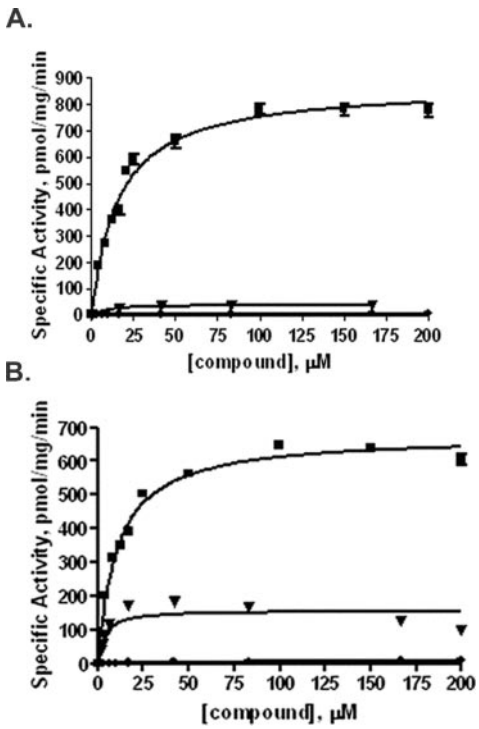

FIG. 4. **Activity of AFC analogs as Icmt substrates.** Total protein (5 μg) from crude His-Ste14p membranes (*A*) or crude His-hIcmt membranes (*B*) were incubated with 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine and increasing concentrations of compounds **1**, **3**, or **4** for 30 min at 30 °C and activity quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments done in duplicate. *Error bars* represent ± 1 S.E. For both panels: ■, **1**; ▼, **3**; ●, **4**.

by this assay (data not shown) (15). *N*-Acetyl-*S*-geranyl-L-cysteine (AGC) is also not an effective His-Ste14p substrate, presumably because of the shorter length of the prenyl moiety (data not shown) (9). In crude membranes, both yeast His-Ste14p (Fig. 4*A*) and human His-hIcmt (Fig. 4*B*) have similar estimated K_m and V_{max} values for the parent compound **1** (Table I). Compound **4** was not a good substrate for either enzyme, demonstrating low V_{max} and relatively high K_m values for the enzymes compared with **1**. Compound **3**, on the other hand, was a substrate for the human enzyme but was a much poorer substrate for the yeast enzyme (Table I and Fig. 4). These results represent some of the first data demonstrating a difference in substrate specificity between the two enzymes.

Compounds **1**, **3**, and **4** were further tested as substrates for purified and reconstituted His-Ste14p (Fig. 5). Purified His-Ste14p was reconstituted in liposomes containing increasing amounts of compound as described previously (14). Unincorporated compound was removed from the liposomes before reconstitution as described under "Experimental Procedures," and activity was measured by the *in vitro* methyltransferase assay (Table II and Fig. 5). The K_m and V_{max} values for these compounds were determined by fitting these curves to the Michaelis-Menten equation. These data demonstrated K_m values for compounds **3** and **4** similar to those obtained for the crude enzyme. Determination of the specificity constants confirmed that both **3** and **4** are much poorer substrates for the pure enzyme than the parent compound **1** (Table II).

AFC Analogs as Inhibitors of His-Ste14p and His-hIcmt—In our assay system, we are unable to distinguish methylation of the inhibitor compound from that of AFC. Therefore, since **3** was a substrate for His-hIcmt (Table I and Fig. 4*B*), we only examined the inhibitory effects of compounds **3** and **4** on His-Ste14p (Fig. 6*A*) and compound **4** on His-hIcmt (Fig. 6*B*) by the *in vitro* vapor diffusion methyltransferase assay. The assays were performed with compound **1** at a concentration of 33 μM, a concentration that is approximately twice its K_m for both His-Ste14p and His-hIcmt in crude membranes. The IC_{50} values for these compounds are reported in Table III. These data also demonstrate a difference in the inhibitory ability of **4** between the yeast and human enzymes. **4** is ~2.5 times less potent of an inhibitor for the human form *versus* the yeast form of Icmt. Inhibition assays were also performed with purified and reconstituted His-Ste14p (Fig. 7). In these experiments, the liposomes containing AFC and inhibitor were not treated to remove unincorporated compound. Because all of the compounds were quantified at a wavelength near 205 nm, there was no way to determine how much of each compound remained in the liposomes after gel filtration, and thus no way to accurately quantify the results. However, the data presented in Fig. 7 follow the same trend of inhibition as that seen for compounds **3** and **4** in crude membranes (Fig. 6*A*).

To characterize the mode of inhibition of Icmt by compounds **3** and **4** and to determine accurate K_I values for the yeast and human enzymes, we performed double-reciprocal

TABLE I
*Compounds **1**, **3**, and **4** as substrates for His-Ste14p and His-hIcmt in crude yeast membrane preparations*

Substrate	His-Ste14p $K_{m(app)}$[a]	His-Ste14p V_{max}	His-hIcmt $K_{m(app)}$[a]	His-hIcmt V_{max}
	μM	*pmol/min/mg*	*μM*	*pmol/mg/min*
1	15.6 ± 0.9	869.6 ± 14.9	10.3 ± 0.5	675.4 ± 7.4
3	8.8 ± 2.2	39.7 ± 1.7	5.4 ± 0.4	200.9 ± 4.5
4	28.0 ± 0.2	5.8 ± 4.4	72.5 ± 5.5	11.2 ± 0.2

[a] K_m values are reported as apparent values because we cannot exactly determine the concentration of substrate accessible to the enzyme.

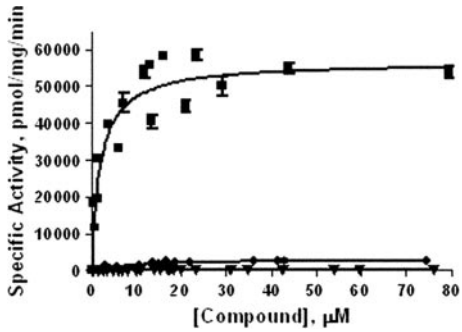

FIG. 5. **AFC analogs as purified His-Ste14p substrates.** Purified His-Ste14p (0.089 μg) was reconstituted in 100 μg of *Escherichia-coli* 100-nm liposomes in the presence of increasing concentrations of compounds **1**, **3**, or **4** as described under "Experimental Procedures." Reactions were carried out for 30 min at 30 °C following addition of 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine. Activity was quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments performed in duplicate. *Error bars* represent ± 1 S.E. ■, **1**; ●, **3**; ▼, **4**.

TABLE II
*Preference of purified, reconstituted His-Ste14p for compounds **1**, **3**, and **4***

Substrate	$K_{m(app)}$ [a]	V_{max}	Specificity constant $k_{cat}/K_{m(app)}$
	μM	*pmol/min/mg*	$M^{-1}\ s^{-1}$
1	2.0 ± 0.2	56,540 ± 999	17,000
3	11.1 ± 1.1	3,266 ± 114	170
4	29.9 ± 3.3	262 ± 14	5.2

[a] K_m values are reported as apparent values because we cannot exactly determine the concentration of substrate accessible to the enzyme.

plot analyses using compound **1** as the substrate. Compound **1** substrate curves were determined by the *in vitro* vapor diffusion methyltransferase assay in the presence of several different fixed concentrations of compound **3** or **4**. For the yeast His-Ste14p enzyme, the double reciprocal plot of these curves shows the characteristic pattern of competitive inhibition for compound **3** (Fig. 8*A*), the expected mode of inhibition for substrate analogs. The double reciprocal plots for compound **4** with both His-Ste14p (Fig. 8*B*) and His-hIcmt (Fig. 8*C*) cross to the left of the *y*-axis, an indication of mixed inhibition, where both a competitive and an uncompetitive component contribute to the inhibition. To obtain K_I values, the substrate curves with different amounts of inhibitor were globally fit to the Michaelis-Menten equation as described under "Experimental Procedures." The K_I values are reported in Table III.

DISCUSSION

The structure-activity relationships presented here give insights into the active site structure of the Icmt family of enzymes. The Icmt family of enzymes are the only known integral membrane methyltransferases, and they lack all of the common binding motifs present in all soluble *S*-adenosylmethionine-dependent methyltransferases (39). In particular, little is known about the nature of the isoprenylated substrate binding site(s). Topology studies demonstrate that Ste14p contains six membrane spans, with the bulk of the protein on the cytosolic side of the endoplasmic reticulum (40). The degree that the enzyme is embedded in the membrane will make crystallization extremely difficult. Therefore, our studies here provide some of the first data characterizing the active site behavior of this family of unusual methyltransferases. Our previous studies focusing on the substrate spec-

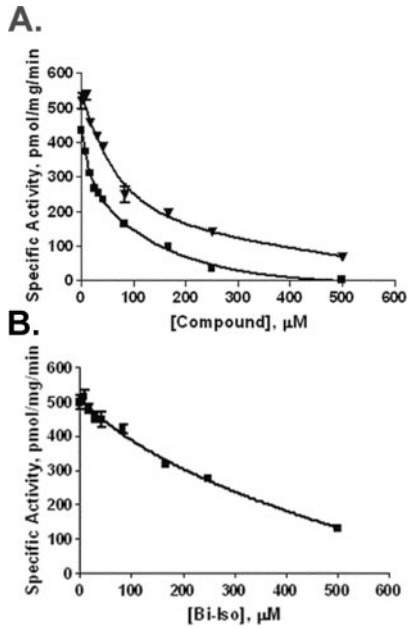

FIG. 6. **AFC analogs as Icmt inhibitors.** *A*, total protein (5 μg) from the crude His-Ste14p membranes were incubated with increasing concentrations of compounds **3** or **4**, 33 μM AFC, and 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine for 30 min at 30 °C and activity quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments done in duplicate. *Error bars* represent ± 1 S.E. ■, **3**; ▼, **4.** *B*, total protein (5 μg) from crude His-hIcmt membranes were incubated with increasing concentrations of compound **4,** 33 μM AFC, and 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine for 30 min at 30 °C and activity quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments done in duplicate. *Error bars* represent ± 1 S.E. ■, **4**.

TABLE III
*Inhibition of Icmt proteins by compounds **3** and **4** in crude membranes*

	IC_{50}	95% Confidence interval	K_I competitive	K_I uncompetitive
	μM	μM	μM	μM
His-Ste14p				
3	44.3	41.1–47.7	17.1 ± 1.7	N/A[a]
4	97.3	86.0–110.0	35.4 ± 3.4	614.4 ± 148
His-hIcmt				
4	259.3	232–290	119.3 ± 18.1	377.2 ± 42.5

[a] Not applicable.

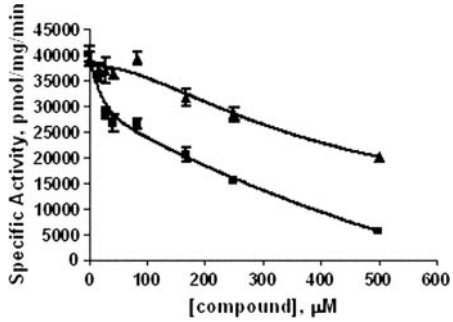

FIG. 7. **AFC analogs as purified His-Ste14p Inhibitors.** Purified His-Ste14p (0.089 μg) was reconstituted in 100 μg of *E. coli* 100-nm liposomes in the presence of increasing concentrations of compounds **3** or **4**, 50 μM AFC, and 20 μM *S*-adenosyl-L-[^{14}C-*methyl*]methionine for 30 min at 30 °C and activity quantified by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." Data are the average of three experiments done in duplicate. *Error bars* represent ± 1 S.E. ■, **3**; ▲, **4**.

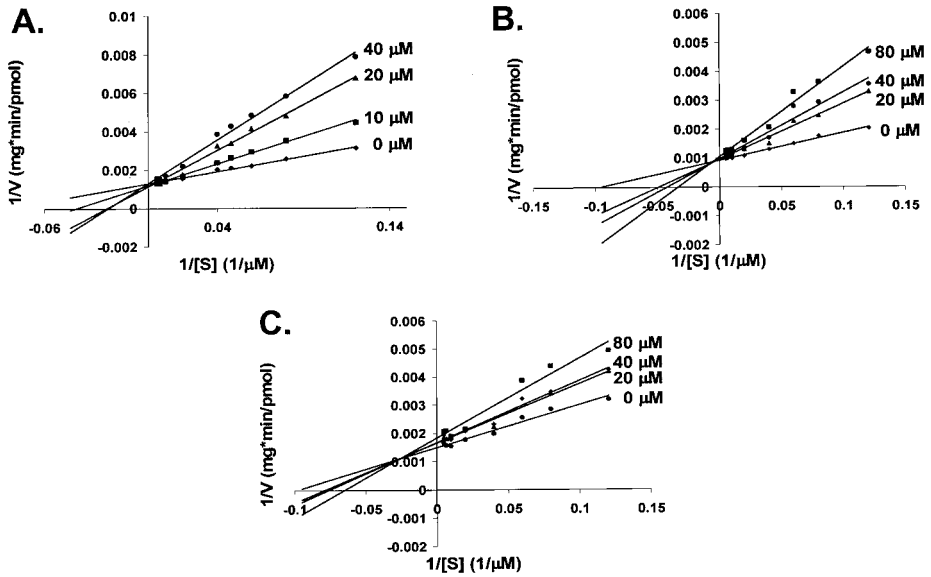

FIG. 8. **Determination of K_I values for compounds 3 and 4.** Activity was determined by the *in vitro* vapor diffusion methyltransferase assay as described under "Experimental Procedures." AFC substrate curves over a 0–200 μM range were performed in the presence of increasing concentrations of **3** or **4** as shown. *A*, Lineweaver-Burk plot for compound **3** with crude His-Ste14p membranes. *B*, Lineweaver-Burk plot for compound **4** with crude His-Ste14p membranes. *C*, Lineweaver-Burk plot for compound **4** with crude His-hIcmt membranes.

ificity of FTase demonstrated that subtle changes in the isoprenoid structure can lead to a complete reversal in biological activity (3, 29, 30, 33, 41). This present study was based on the hypothesis that a related pattern might emerge from a similar study of Icmt.

The ability of an AFC analog to act as a substrate appears to be influenced by a large substituent in the 3-position of the farnesyl group, such as the isobutenyl moiety in compound 3. This compound has the ability to bind to the enzyme well but exhibits a low turnover, making it an ideal candidate for a lead His-Ste14p inhibitor. However, this analog is a relatively good substrate for the human Icmt enzyme. The observation that compound **3** is a better substrate for His-hIcmt than for His-Ste14p relative to compound **1** suggests that the human enzyme is more tolerant of substitution in the farnesyl moiety.

The compound with the lowest V_{max}, **4**, combines the isobutenyl substitution at position 3 with the two phenyl rings in place of the last two prenyl moieties. These perturbations to the isoprene chain reduce its ability to act as a substrate by both raising the K_m and lowering the V_{max} substantially. It is interesting to note that the increased binding affinity observed with compound **3** because of the 3-isobutenyl substitution is offset in **4** by the presence of the biphenyl group.

Our two inhibitors, compounds **3** and **4**, both have an isobutenyl substituent on the 3 position of the isoprenyl moiety, further emphasizing the importance of this position in recognition by Icmt. Because we found compounds **3** and **4** to be competitive and mixed inhibitors respectively, the presence of the substituent at this position on the isoprene could possibly cause the compound to bind in the active site in such a way that results in slower release as compared with the parent compounds. The lead compounds **3** and **4** described here are among the first well characterized inhibitors of Icmt. These compounds, reversible competitive or mixed inhibitors with K_m values comparable to the parent compound **1** but low V_{max} values, would release significantly lower amounts of the methylated prenylcysteine product when used *in vivo* to inhibit Icmt and may allow for lower dosing than AFC, which is currently used in cellular studies to determine the cellular consequences of prenylcysteine methylation (42).

The effects of Icmt-mediated methylation on signal transduction represent an area of growing interest and significant therapeutic promise (42–44). Recently, another small molecule inhibitor for Icmt, cysmethynil, has been identified from a large chemical library (43). This compound causes mislocalization of Ras and blocks anchorage-independent growth of a human colon cancer cell line, further validating the importance of Icmt as a molecular target for further drug development. The reported IC_{50} for this compound for the *in vitro* inhibition of Icmt activity was 0.2–2.4 μM (43). When tested under similar assay conditions, compound **3** described in this study demonstrated an IC_{50} of 7.3–14.5 μM for His -Ste14p (data not shown).

Together with the recent biological studies that demonstrated that inhibition of Icmt results in the mislocalization and loss of transforming ability of K-Ras (19), these small molecule inhibition studies suggest that Icmt is an attractive anticancer target. The further development and refinement of pharmacological tools should not only provide a means to study the mechanism of action of Icmt but also will provide a means to further evaluate Icmt as a chemotherapeutic target.

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