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NOVEL INHIBITORS OF THE PRENYLATED PROTEIN METHYLTRANSFERASE REVEAL DISTINCTIVE STRUCTURAL REQUIREMENTS

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Abstract: Inhibitors of a prenylated protein methyltransferase were synthesized and evaluated. S-farnesyl-5-fluorothiosalicylic acid and the 5-chloro analog (but not the 4-fluoro, 4-chloro or 3-chloro analogs) were potent inhibitors, as was the parent compound S-farnesyl thiosalicylic acid (FTS), whose methyl ester was far less active. S-geranyl and S-geranylgeranyl thiosalicylic acids were more than ten times less potent than FTS.
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Introduction

The enzyme prenylated protein methyltransferase (PPMTase) methylates the free carboxyl group in the C-terminal S-prenyl cysteine of a variety of proteins, including proteins involved in signal transduction such as Ras, Rac, Rho and Gβγ of the heterotrimeric G-proteins.[1,2] Methylation of these proteins by PPMTase appears to play an important modulatory role by increasing their membrane association and in several cases their interactions with effector molecules, both of which are required for productive signal transduction.[2,3,4] Therefore, PPMTase inhibitors can serve as useful signal transduction interceptors, and as specific tools for studies on the interactions of prenylated proteins with their target sites and with the PPMTase.

We previously showed that the rigid structure of thiosalicylic acid provides an appropriate backbone for the preparation of PPMTase inhibitors such as S-trans,trans farnesyl thiosalicylic acid.[4,5] Based on these results we have prepared novel S-prenyl derivatives of thiosalicylic acid (Table 1), and demonstrate here that the length of the S-prenyl group and the nature and locality of halogenic substituents on the benzene ring and the free carboxyl group determine their inhibitory potency.

Enzyme assays

Each of the new compounds was tested for its ability to serve as a substrate or as an inhibitor for PPMTase, using an *in vitro* assay described in detail previously.[6,7] Briefly, rat cerebellar synaptosomes (125 μg protein) were incubated in Tris buffer pH 7.4 containing 8% dimethylsulfoxide, 25 μM [methyl-^{3}H]-S-adenosyl-L-

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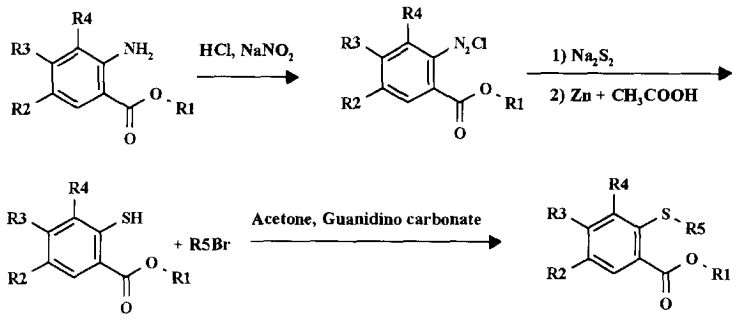
methionine, 25 μM N-acetyl-S-farnesyl-L-cysteine (AFC, methyl acceptor) and the indicated concentrations of an S-prenyl analog. For substrate activity AFC was omitted. Following incubation for 10 min at 37°C, the AFC [^{3}H]-methyl esters formed were extracted by chloroform/methanol, base-hydrolyzed. The [^{3}H]-methanol thus formed was estimated by vapor-phase diffusion assays.[6,7] Other procedures, where hexane or heptane were used to extract products, yielded results similar to those described here.

Results & Discussion

The aromatic ring of FTS is amenable to relatively simple synthetic modifications like substitution at different positions, with substituents having different electronegativities and sizes (*e.g.* H, Cl, F). Moreover, we modified the prenyl radical (farnesyl, geranyl or geranylgeranyl) in order to evaluate the importance of the prenylthio substituent. Finally, we prepared the carboxy methyl ester of FTS (FTS-Me) to determine whether or not the proton of the carboxylic acid moiety is needed for the interaction with the PPMTase. All of the S-prenyl derivatives were prepared in a three-step process presented schematically in Scheme I. The anthranilic acid derivatives were converted to the corresponding thiosalicylic acid derivatives according to a known procedure.[8] The last step, giving rise to new prenyl derivatives, was performed at room temperature (except in the case of 3-Cl-FTS, performed at 60°C) by alkylation of the mercapto group with different alkylating prenyl halides (farnesyl, geranyl or geranylgeranyl bromides). A typical synthetic procedure together with the physical data of all the prenyl derivatives is described.[9] Table 1 lists the S-prenyl derivatives prepared.

Scheme I.



Initial experiments indicated that the new S-prenyl derivatives could not serve as substrates for the PPMTase. Thus, unlike in non-rigid derivatives such as AFC or S-farnesylthiopropionic acid (FTP) where the free carboxyl groups are methylated by PPMTase,[2,7] the free carboxyl groups of the S-prenyl thiosalicylic acid derivatives cannot serve as methyl acceptors. On the other hand, FTS (**1**) and its 5-fluoro (**2**) and 5-chloro (**3**) derivatives appeared to be rather potent inhibitors of the PPMTase (Fig. 1), with estimated Ki values in the μM range (Table

1). Halogenic substitutions at position 4 of the benzoic ring, as in 4-F-FTS (**4**) and 4-Cl-FTS (**5**), caused a marked decrease in potency, and substitution at position 3 yielded a compound, *e.g.*, 3-Cl-FTS (**6**), which was inactive even at a concentration of 75 μM (Fig. 1, Table 1).

Table 1. New PPMTase inhibitors, their Ki values (μM) and synthetic data

	R1	R2	R3	R4	R5	T	Yield* (%)	Ki μM
(1) FTS**	H	H	H	H	Farnesyl	RT	85	2.8± 1.3
(2) 5-F-FTS	H	F	H	H	Farnesyl	RT	18	6.3± 3.1
(3) 5-Cl-FTS	H	Cl	H	H	Farnesyl	RT	13	8.5±2.4
(4) 4-F-FTS	H	H	F	H	Farnesyl	RT	13	23.0±2.7
(5) 4-Cl-FTS	H	H	Cl	H	Farnesyl	RT	18	28.0±3.0
(6) 3-Cl-FTS	H	H	H	Cl	Farnesyl	60°C	10	> 50
(7) FTS-Me	CH_3	H	H	H	Farnesyl	RT	14	> 50
(8) GTS	H	H	H	H	Geranyl	RT	37	>50
(9) GGTS	H	H	H	H	Geranylgeranyl	RT	79	> 50

* T = reaction temperature; Yield of the last step. The yields were not optimized. ** Previously described[5]. The Ki's were calculated from the IC_{50}'s determined in the inhibition curves as shown in Fig. 1 using the equation $K_i = IC_{50} / (1+S/K_M)$. The given values represent the means ± SD of three separate experiments.

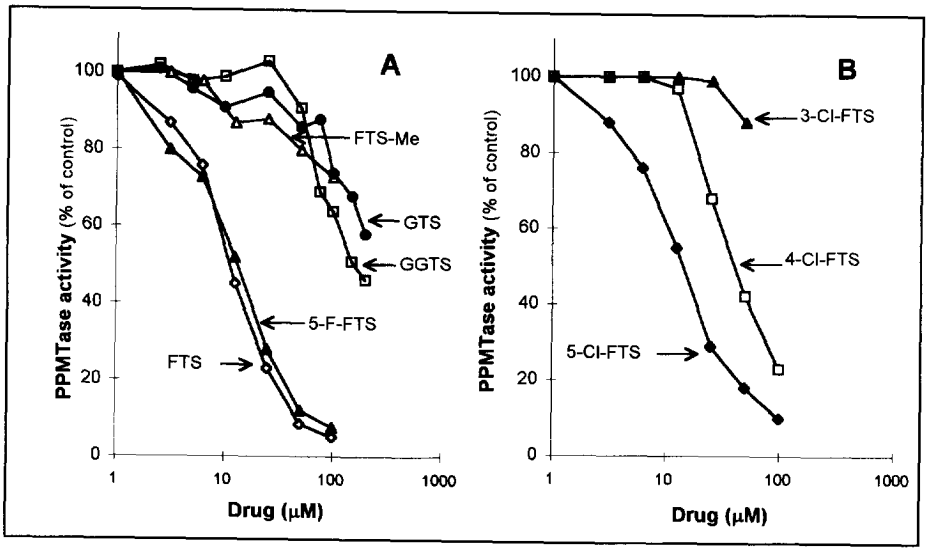


Fig. 1. Inhibition of PPMTase by S-prenyl derivatives. PPMTase activity was determined with 25 μM AFC in the absence (control) and in the presence of the indicated concentrations of the drugs.

Although the free carboxyl group in the prenyl derivatives of thiosalicylic acid does not serve as a methyl acceptor for the PPMTase, it appears to be important for interaction with the enzyme, as indicated by the fact that FTS-Me (7) did not yield 50% inhibition of PPMTase activity even at a concentration of 100 μM (Fig. 1). In addition to the free carboxylic group, the length of the prenyl moiety is critical: the C_{10} geranyl derivative, GTS (8), did not inhibit the PPMTase at a concentration of up to 75 μM, and the C_{20} geranylgeranyl derivative, GGTS (9), was a weak inhibitor with an K_i value of 75 μM (Fig. 1, Table 1). It is important to note that all compounds exhibiting inhibitory activity were competitive with respect to the methyl acceptor substrate AFC. Typical examples of such competitive inhibitions are shown in Figure 2.

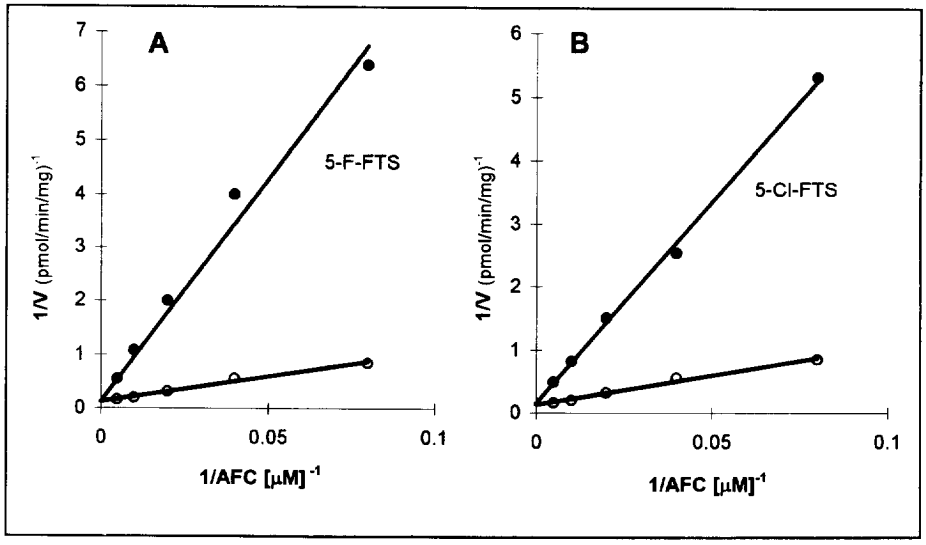


Fig. 2. Competitive inhibition of the PPMTase by 5-Cl-FTS and by 5-F-FTS. Enzyme activity was determined (with various concentrations of AFC) in the absence (o) or in the presence (o) of 50 μM 5-F-FTS (A) or 50 μM 5-Cl-FTS (B). Data are presented in the form of double reciprocal plots.

Taken together, the structure-activity relationships described here define the rather stringent structural requirements for inhibition of the PPMTase by S-prenyl derivatives of thiosalicylic acid. It appears that for this activity the C_{15} farnesyl group is preferred over the C_{10} geranyl or C_{20} geranylgeranyl group, a free carboxyl group is absolutely required, and halogenic substitutions at position 5 of the benzene ring (but not at positions 3 or 4) are tolerated. We suggest that these requirements reflect the nature of the prenylated substrate/inhibitor-binding site of the PPMTase. The much lower affinity of GGTS than of FTS may reflect the preference of the

PPMTase preparation used in our experiments for farnesylated substrates. If so, a PPMTase that appears to prefer geranylgeranylated substrates[10] may be inhibited by GGTS.

In conclusion, the new PPMTase inhibitors described here can serve as specific tools for the characterization and purification of PPMTase subtypes and may prove useful as specific signal transduction interceptors that would differentially affect the activity of farnesylated or of geranylgeranylated proteins. We know from earlier studies[5,6] that the parent compound FTS affects the functions of oncogenic Ras proteins. It will be interesting to see whether the new derivatives can mimic these effects of FTS.

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9. Compounds **(2)-(9)** were synthesized according to the following procedure. The thiosalicylic acid derivative, guanidino carbonate and the appropriate alkenyl bromide in the molar ratio (1:1.1:1) in dry acetone (about 15 ml/mmol) were mixed overnight at room temperature, protected from light. After the acetone was evaporated, chloroform was added together with 2N HCl, which brings the pH of the solution to 2-3. The organic phase was separated and washed with water, dried on magnesium sulfate and evaporated. The product was purified by column chromatography on silica gel. The eluting mixtures used depend on the product and are as follows: 5-F-FTS: chloroform - ethyl acetate (5:1-1:5); 5-Cl-FTS: chloroform - ethyl acetate (1:1) to ethyl acetate; 4-F-FTS: chloroform-hexane to chloroform; 4-Cl-FTS: chloroform - hexane (19:1) to chloroform; 3-Cl-FTS: hexane - ethyl acetate (98:2 - 85:15) [overnight reflux

at 60°C]; FTS-Me: The pasty compound precipitates from hexane (no chromatography); GTS: chloroform - ethyl acetate (9:1-7:3); GGTS: chloroform- ethyl acetate (1:9).

5-F-FTS: 5-Fluoro-2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoic acid. Yellowish solid. ^{1}H-NMR ($CDCl_3$) δ 1.6(bs,6H), 1.7 (2s,6H), 2.1(m,8H), 3.6(d,2H), 5.1(m,2H), 5.35 (bt, 1H), 7.3 (m,1H, Arom), 7.43 (m,1H, Arom), 8.1 (m,1H, Arom) ppm. High resolution MS m/e 376 (M^+) ($C_{22}H_{29}O_2SF$).

5-Cl-FTS: 5-Chloro-2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoic acid. Pale yellowish oil. ^{1}H-NMR ($CDCl_3$) δ 1.6(bs,9H), 1.65 (s,3H), 2.1(m,8H), 3.6(d,2H), 5.1(m,2H), 5.3(bt, 1H), 7.2 (m,1H, Arom), 7.4 (m,1H, Arom), 7.9(m,1H, Arom) ppm. High resolution MS m/e 394, 392 (M^+) ($C_{22}H_{29}O_2SCl$).

4-F-FTS: 4-Fluoro-2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoic acid. ^{1}H-NMR ($CDCl_3$) δ 1.6(s,6H), 1.71 (d,6H), 2.1(m,8H), 3.56(d,2H), 5.09(m,2H), 5.33(bt, 1H), 6.86 (m,1H, Arom), 7.0 (m,1H, Arom), 8.15(m,1H, Arom) ppm.

4-Cl-FTS: 4-Chloro-2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoic acid. Pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ 1.6(s,9H), 1.74 (d,6H), 2.1(m,8H), 3.6(d,2H), 5.1(bt,2H), 5.3(bt, 1H), 7.16 (m,1H, Arom), 7.29 (m,1H, Arom),8.06 (m,1H, Arom) ppm. High resolution MS m/e 394, 392 (M^+) ($C_{22}H_{29}O_2SCl$).

3-Cl--FTS: 3-Chloro-2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoic acid. Yellowish oil. ^{1}H-NMR ($CDCl_3$) δ 1.6(s,3H), 1.62 (s,3H), 1.64 (s,3H), 1.8 (s,3H), 1.96-2.2 (m,8H), 4.8 (d,2H), 5.1(m,2H), 5.45 (m, 1H), 6.58 (t,1H, Arom), 7.38 (d, 1H, Arom), 7.84 (d, 1H, Arom) ppm.

FTS-Me: Methyl 2-[(3,7,11-trimethyl-dodeca-2,6,10-trienyl)sulfanyl]benzoate. Yellowish paste. ^{1}H-NMR ($CDCl_3$) δ 1.6(s,9H), 1.65 (2s,6H), 2.1(m,8H), 3.47(s,3H), 3.6 (d, 2H), 5.1(bt,2H), 5.35(bt, 1H), 7.2 (m,1H, Arom), 7.35 (m,1H, Arom), 7.45(m,1H, Arom), 8.15 (m, 1H, Arom) ppm. High resolution MS m/e 372 (M^+) ($C_{23}H_{32}O_2S$).

GTS: 2-[(3,7-dimethyl-octa-2,6-dienyl)sulfanyl]benzoic acid. ^{1}H-NMR ($CDCl_3$) δ 1.60(s,3H), 1.65 (s,3H), 1.67 (s, 3H), 2.02 (m,4H), 3.58(d,2H), 5.06 (m,2H), 5.33(m, 1H), 7.22 (m,1H, Arom),7.39 (m,1H, Arom) 7.47 (m,1H,Arom), 8.15(m,1H, Arom) ppm.

GGTS: 2-[(3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)sulfanyl]benzoic acid. ^{1}H-NMR ($CDCl_3$) δ 1.60(s,9H), 1.66 (s,3H), 1.67 (s, 3H), 2.02 (m,4H), 2.05(m, 8H), 3.58(d,2H), 5.10 (m,2H), 5.33(m, 1H), 7.20 (m,1H, Arom),7.38 (m,1H, Arom) 7.47 (m,1H,Arom), 8.14(m,1H, Arom) ppm.

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