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Synthesis of aniline-type analogues of farnesyl diphosphate and their biological assays for prenyl protein transferase inhibitory activity

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Abstract

Stable analogues of farnesyl diphosphate, possessing an aniline-type portion in the prenyl-mimic moiety and phosphonoacetamido(oxy) groups in the place of the metabolically unstable diphosphate unit, were synthesised and submitted to biological assays. The enzyme inhibition tests performed on FTase and GGTase I show that the newly synthesised compounds based on a combination of the aniline-containing portions with (phosphonoacetamido)oxy groups do not afford potent inhibitors.

Keywords: Farnesyldiphosphate; FTase; Aniline; Hexafluoroaniline; Phosphonoacetamido(oxy)

1. Introduction

Small G-proteins belonging to the Ras superfamily are actively involved in signal transduction processes. These proteins are initially activated by an isoprenylation which makes them more lipophilic and, therefore, capable of anchoring to the inner part of cellular membrane, where the remaining activation of signal transduction takes place [1]. The isoprenylation process is carried out mostly by a farnesyl-protein transferase (FTase) which uses farnesyldiphosphate (FdP) as the isoprenylating portion, or by a geranylgeranyl-protein transferase (GGTaseI) which uses geranylgeranyldiphosphate (GGdP). The observation that inhibitors of the enzyme hydroxymethylglutaryl-CoA reductase (designed as a cholesterol-lowering agent that reduces the production of isoprenylating intermediates), also reduced cell proliferation [2], suggested a possible application in cancer therapy of molecules able to block the prenylation of Ras proteins, by means of the inhibition of the key enzymes responsible for this process (FTase, GGTaseI) [3,4]. So far the most widely studied target enzyme has undoubtedly been FTase, for which inhibitors have been designed as: (i) carboxy-terminal (CAAX-motif) mimics of Ras proteins; (ii) FdP analogues; and (iii) "dual mimics" possessing molecular portions similar to both the CAAX-motif and the FdP structure [5].

Recently, new farnesyl side chain mimics were identified in an aniline-type moiety [6]. In fact compound **1** (Fig. 1) proved to be an inhibitor of FTase with an IC_{50} value (0.50 μM) comparable to the natural substrate FdP. Moreover, **1** proved to be inactive on GGTase I ($IC_{50} > 20$ μM), thus showing a good FTase over GGTase selectivity [6]. Another efficient farnesyl mimic group was found in a pentafluoroaniline-type portion, as shown in compound **2** (Fig. 1), which proved to be effectively recognized by FTase [7].

The presence of a diphosphate unit in compounds **1** and **2** makes them metabolically unstable and, therefore, hardly suitable for future development. As a matter of fact, di-phosphate derivatives are quickly hydrolysed by

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phosphoesterase enzymes, before they can exert their pharmacological action. We therefore focused our attention on modifying the polar portion of compounds of type **1** with diphosphate mimics, stable to phosphoesterases, which could afford "drug-like" molecules. We considered the (phosphonoacetamido)oxy portion, already successful in a previously developed FTase inhibitor (**FTP II**, Fig. 1) [8], and its ethyl-substituted analog, as appropriate candidates.

In an attempt to generate more potent and selective FTase inhibitors, we designed and synthesised compounds **3a**,**b** and **4a**,**b** (Fig. 1) deriving from a combination of the isoprenyl-mimic portion of **1** and **2** and the polar portion, or its ethyl-substituted analog, of compound **FTP II**.

2. Chemistry

Compounds **3a**,**b** and **4a**,**b** were synthesized as shown in Scheme 1. Allylic alcohols **5** [6] or **6** [7] were submitted to a Mitsunobu reaction with *N*-hydroxyphthalimide to give phthalimide derivatives **7** and **8**. Hydrazinolysis of the phthalimido portion afforded free oxyamines **9** and **10**, respectively. A subsequent condensation reaction, promoted by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole, with diethylphosphonoacetic acid (**11a**) afforded diethylphosphonates **12a** and **13a**, which were submitted to an ester hydrolysis reaction involving an initial treatment with bromotrimethylsilane and 2,4,6-collidine, followed by aqueous potassium hydroxide, to give compounds **3a** and **4a** as the dipotassium salts.

Condensation of oxyamines **9** and **10** with 2-(diethylphosphono)butyric acid (**11b**) [9], under the same conditions described above, afforded the ethyl-substituted diethylphoshonates **12b** and **13b**. Final ester hydrolysis of **12b** and **13b**, using the same procedure described for **12a** and **13a**, afforded **3b** and **4b** as the dipotassium salts.

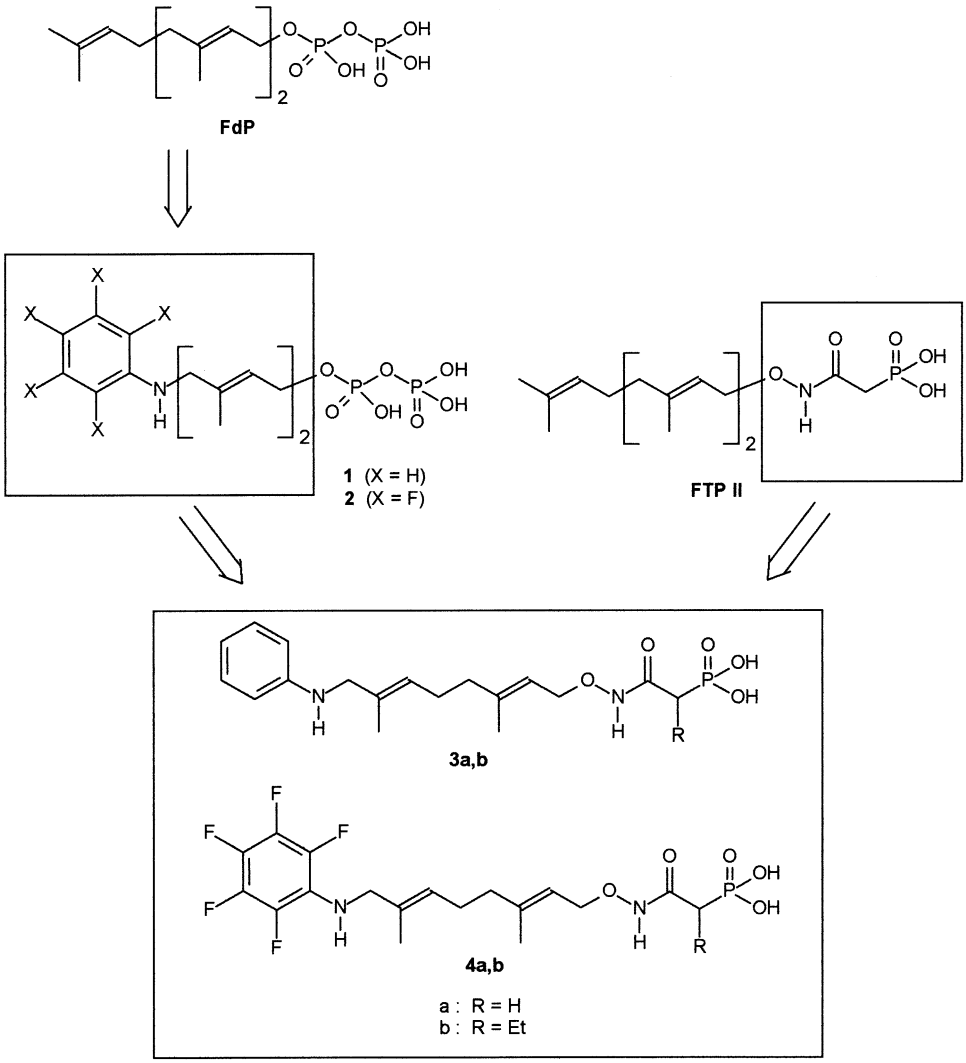
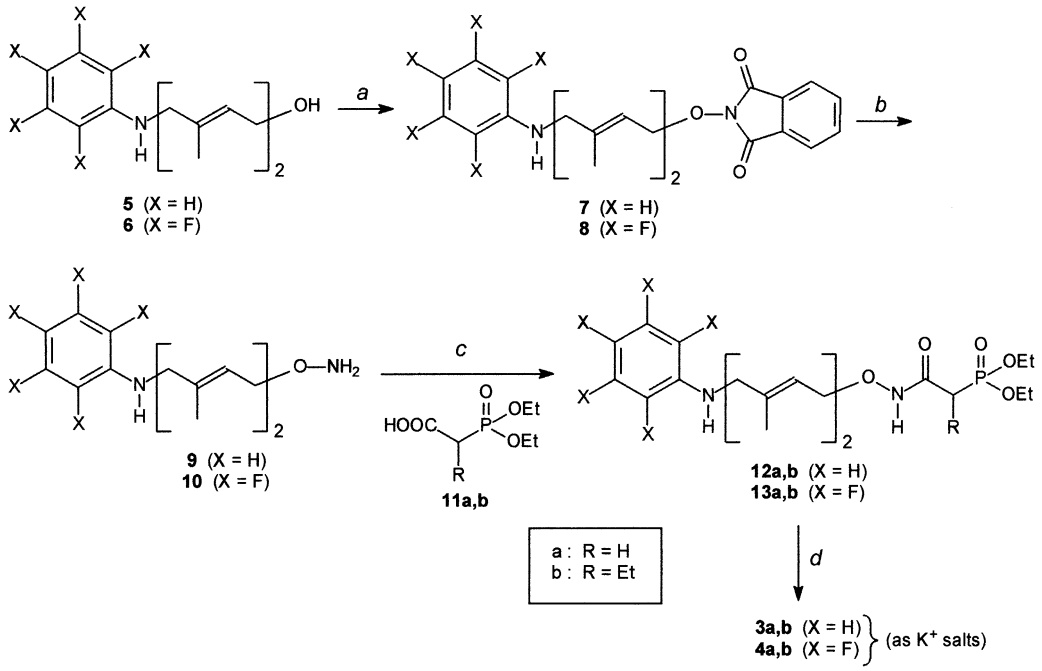


Fig. 1. Structural derivation of FPP analogues **3a**,**b** and **4a**,**b**.



Scheme 1. [a]Reagents and conditions: (a) *N*-Hydroxyphthalimide, Ph_3P, DEAD, THF, r.t. (b) $NH_2NH_2 \cdot H_2O$, EtOH, r.t. (c) 1-Hydroxybenzotriazole, EDC, THF, r.t. (d) 1) TMS-Br, 2,4,6-collidine, CH_2Cl_2, r.t; 2) 1N aqueous KOH.

Table 1
Inhibitory activity of compounds **3a**,**b** and **4a**,**b** on farnesyl protein transferase (FTase) and geranylgeranyl protein transferase I (GGTase I), together with a reference FTase inhibitor **FTP II** and aniline derivative **1**

Comp.	IC_{50} (μM) [a]	
	FTase	GGTase I
1 [b]	0.5	> 20
FTP II [c]	0.043 ± 0.0045	> 10.0
3a	> 10.0	> 10.0
3b	3.5 ± 0.4	> 10.0
4a	> 10.0	4.5 ± 0.6
4b	> 10.0	8.5 ± 1.0

[a] Values are reported as the mean ± range or SD of 2–3 independent experiments.
[b] Reference [6].
[c] Reference [8].

3. Results and discussion

The in vitro inhibition assays of FTase and GGTase I were carried out by measuring the [^{3}H]GGdP and [^{3}H]FdP incorporated into H-Ras-CVLL and H-Ras-CVLS, respectively, as described in Section 4.2.2. The activity of the inhibitors is reported in Table 1 as their IC_{50}, the concentration at which FTase and GGTase I activity was inhibited by 50%. As reference compounds of this class of structures, we have also reported the inhibitory properties of **FTP II** [8] and aniline derivative **1** [6].

An analysis of the results shows that none of the new compounds is endowed with any remarkable inhibition potencies on either of the two enzymes tested. The only compound showing a certain inhibition of FTase ($IC_{50} = 3.5$ μM) was aniline-type derivative **3b**, which possesses an ethyl substituent on its phosphonoacetamido(oxy) portion. This level of inhibition, albeit modest, is accompanied by a total absence of GGTaseI inhibition ($IC_{50} > 10$ μM) which confers on **3b** a certain FTase selectivity.

The introduction of five fluorine atoms in the aromatic portions, as in compounds **4a** and **4b**, caused a complete loss of the FTase inhibitory properties. Compound **4a** showed some activity on GGTase I, whereas **4b** is almost inactive also on this enzyme.

In conclusion, we have designed and synthesised new stable aniline-type FdP-analogues. The biological results show that the combination of the aniline-containing portions as farnesyl side chain mimics [6,7], together with (phosphonoacetamido)oxy groups [8] as diphosphate stable mimics, do not afford potent inhibitors of the FTase enzyme.

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra of all

compounds were obtained with a Varian Gemini-200 instrument operating at 200 MHz; the data are reported as follows: chemical shift (in ppm) from the Me_4Si line as the external standard, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, m = multiplet). Mass spectra were recorded on a VG 70-250S mass spectrometer or a HP-5988 A spectrometer. Analytical TLCs were carried out on 0.25-mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatography was performed using 230–400 mesh silica gel. Sodium sulfate was always used as the drying agent. Evaporations were performed in vacuo (rotating evaporator). Commercially available chemicals were purchased from Sigma–Aldrich.

4.1.2. Synthesis of derivatives 7 and 8

A solution containing 4.1 mmol of the appropriate precursor (**5** [6] or **6** [7]) in 50 ml of anhydrous THF was treated with *N*-hydroxyphthalimide (1.4 g, 8.8 mmol), triphenylphosphine (2.6 g, 9.9 mmol) and diethylazodicarboxylate (1.6 ml, 9.9 mmol) and the resulting mixture was stirred for 24 h at r.t. under an inert atmosphere. The solvent was then evaporated under a vacuum and the residue was purified by column chromatography on 230–400 mesh silica gel eluting with Hex/CH_2Cl_2. The appropriate fractions were combined and evaporated to give the corresponding products.

7 (1.45 g, 97%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.64 (s, 3H), 1.71 (s, 3H), 2.03–2.28 (m, 4H), 3.61 (s, 2H), 4.71–4.75 (m, 2H), 5.35–5.56 (m, 2H), 6.57–6.71 (m, 3H), 7.11–7.19 (m, 2H), 7.70–7.93 (m, 4H). MS (EI, 70eV): *m*/*z* 366 (M^+).

8 (1.86 g, 94%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.62 (s, 3H), 1.71 (s, 3H), 2.06–2.15 (m, 4H), 3.76 (s, 2H), 4.71–4.75 (m, 2H), 5.27–5.56 (m, 2H), 7.71–7.85 (m, 4H). MS (EI, 70eV): *m*/*z* 480 (M^+).

4.1.3. Synthesis of derivatives 9 and 10

Hydrazine monohydrate (1.5 ml, 31 mmol) was added to a solution containing 3.8 mmol of the appropriate precursor (**7** or **8**) in EtOH (60 ml) and the resulting mixture was stirred at r.t. for 40 h. After removal upon filtration of the white solid formed, the resulting solution was evaporated and the oily crude residue was purified by column chromatography on 230–400 mesh silica gel eluting with CH_2Cl_2/Et_2O. The appropriate fractions were combined and evaporated to give the corresponding products.

9 (0.90 g, 91%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.67 (s, 6H), 2.05–2.23 (m, 4H), 3.63 (s, 2H), 4.16–4.21 (m, 2H), 5.34–5.42 (m, 2H), 6.57–6.72 (m, 3H), 7.12–7.20 (m, 2H). MS (EI, 70eV): *m*/*z* 260 (M^+).

10 (1.2 g, 89%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.65 (s, 3H), 1.66 (s, 3H), 2.01–2.18 (m, 4H), 3.79 (s, 2H), 4.14–4.19 (m, 2H), 5.25–5.40 (m, 2H). MS (EI, 70eV): *m*/*z* 350 (M^+).

4.1.4. Synthesis of derivatives 12a and 13a

A solution containing 0.83 mmol of the appropriate precursor (**9** or **10**), diethylphosphonoacetic acid **11a** (0.180 g, 0.92 mmol), and 1-hydroxybenzotriazole (0.170 g, 1.25 mmol) in anhydrous THF (15 ml) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.190 g, 0.996 mmol). The mixture was stirred at r.t. for 24 h, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography on 230–400 mesh silica gel eluting with CH_2Cl_2/acetone (9:1). The appropriate fractions were combined, evaporated, and pump-dried to give the corresponding product.

12a (0.16 g, 44%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.36 (t, 6H, J = 7.1 Hz), 1.66 (s, 3H), 1.70 (s, 3H), 2.00–2.15 (m, 4H), 2.82 (d, 2H, J = 20 Hz), 3.64 (s, 2H), 3.95 (br, 1H), 4.05–4.20 (m, 4H), 4.41–4.46 (m, 2H), 5.29–5.39 (m, 2H), 6.61–6.74 (m, 3H), 7.12–7.20 (m, 2H), 9.50 (br, 1H). MS (EI, 70eV): *m*/*z* 438 (M^+).

13a (0.16 g, 37%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, 6H, J = 7.1 Hz), 1.65 (s, 3H), 1.72 (s, 3H), 1.97–2.17 (m, 4H), 2.84 (d, 2H, J = 20 Hz), 3.80 (s, 2H), 4.09–4.24 (m, 4H), 4.42–4.45 (m, 2H), 5.30–5.42 (m, 2H), 9.57 (br, 1H). MS (EI, 70eV): *m*/*z* 528 (M^+).

4.1.5. Synthesis of derivatives 12b and 13b

A solution containing 0.83 mmol of the appropriate precursor (**9** or **10**), was submitted to the same treatment described above (Section 4.1.4) using 2-(diethylphosphono)butyric acid **11b** [9] (0.205 g, 0.92 mmol). The crude product was purified by column chromatography on 230–400 mesh silica gel eluting with hexane/EtOAc (2:8). The appropriate fractions were combined, evaporated, and pump-dried to give the corresponding product.

12b (0.12 g, 30%) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.02 (t, 3H, J = 7.1 Hz), 1.40 (t, 6H, J = 7.0 Hz), 1.62 (s, 3H), 1.67 (s, 3H), 1.73–2.15 (m, 7H), 3.58 (s, 2H), 3.92 (br, 1H), 4.03–4.16 (m, 4H), 4.40–4.44 (m, 2H), 5.28–5.40 (m, 2H), 6.60–6.75 (m, 3H), 7.08–7.15 (m, 2H). MS (EI, 70eV): *m*/*z* 466 (M^+).

13b (0.21 g, 45%) as an oil; 1.00 (t, 3H, J = 7.1 Hz), 1.32 (t, 6H, J = 7.0 Hz), 1.63 (s, 3H), 1.68 (s, 3H), 1.86–2.16 (m, 7H), 3.77 (s, 2H), 4.10–4.21 (m, 4H), 4.36–4.40 (m, 2H), 5.29–5.36 (m, 2H). MS (EI, 70eV): *m*/*z* 556 (M^+).

4.1.6. Synthesis of the dipotassium salts of derivatives 3a and 4a

A solution containing 0.25 mmol of the appropriate precursor (**12a** or **13a**), was treated with bromotrimethylsilane (0.17 ml, 1.2 mmol) and 2,4,6-collidine (0.07 ml, 0.5 mmol) in anhydrous CH_2Cl_2 (3 ml); the

resulting mixture was stirred at r.t. for 18 h. After concentration of the solution, the residue was treated with an aqueous solution of KOH 1 N (3 ml) and then stirred at r.t. for 4 h. The solution was evaporated and the resulting crude residue was purified by column chromatography on reverse phase silica gel (Merck Lichroprep® RP-18) eluting with MeOH/H_2O (6:4). The appropriate fractions were combined, evaporated, lyophilised, and pump-dried to give the corresponding compound as the dipotassium salt.

3a·$2K^+$ (0.090 g, 78%) as a solid; ^{1}H NMR (D_2O) δ 1.54 (s, 3H), 1.60 (s, 3H), 1.94–2.10 (m, 4H), 2.28 (d, 2H, $J = 19$ Hz), 3.56 (s, 2H), 4.22–4.28 (m, 2H), 5.28–5.34 (m, 2H), 6.69–6.78 (m, 3H), 7.12–7.22 (m, 2H). MS (FAB): m/z 459 ($M+H^+$).

4a·$2K^+$ (0.13 g, 92%) as a solid; ^{1}H NMR (D_2O) δ 1.37 (s, 3H), 1.43 (s, 3H), 1.78–1.88 (m, 4H), 2.33 (d, 2H, $J = 19$ Hz), 3.48 (s, 2H), 4.16–4.20 (m, 2H), 5.08–5.16 (m, 2H). MS (FAB): m/z 549 ($M+H^+$).

4.1.7. Synthesis of the dipotassium salts of derivatives *3b* and *4b*

A solution containing 0.25 mmol of the appropriate precursor (**12b** or **13b**), was submitted to the same treatment described above (Section 4.1.6). The crude product was purified by column chromatography on reverse phase silica gel (Merck Lichroprep® RP-18) eluting with MeOH/H_2O (1:1). The appropriate fractions were combined, evaporated, lyophilised, and pump-dried to give the corresponding compound as the dipotassium salt.

3b·$2K^+$ (0.067 g, 55%) as a solid; ^{1}H NMR (D_2O) δ 0.66 (t, 3H, $J = 7.1$ Hz), 1.32 (s, 3H), 1.40 (s, 3H), 1.58 (q, 2H, $J = 7.3$ Hz), 1.70–2.08 (m, 5H), 3.30 (s, 2H), 4.11–4.15 (m, 2H), 5.07–5.13 (m, 2H), 6.40–6.52 (m, 3H), 6.87–6.95 (m, 2H). MS (FAB): m/z 489 ($M+H^+$).

4b·$2K^+$ (0.11 g, 80%) as a solid; ^{1}H NMR (D_2O) δ 0.62 (t, 3H, $J = 7.1$ Hz), 1.32 (s, 3H), 1.37 (s, 3H), 1.55 (q, 2H, $J = 7.0$ Hz), 1.68–2.06 (m, 5H), 3.43 (s, 2H), 4.10–4.13 (m, 2H), 4.99–5.13 (m, 2H). MS (FAB): m/z 577 ($M+H^+$).

4.2. Biological assays

4.2.1. Materials

[^{3}H]GGPP (specific activity 22 Ci/mmol) and [^{3}H]FdP (specific activity 16 Ci/mmol) were purchased from Perkin–Elmer Life Science; GGTase I and FTase were purchased from Sigma-Aldrich Corporation; H-Ras-CVLL, H-Ras-CVLS (Wild type), FPT inhibitor II (**FTP II**) [8] and Zwittergent 3-12 were purchased from Calbiochem. All other reagents were obtained from normal commercial sources.

4.2.2. GGTase I and FTase activity assay

In vitro inhibition studies were performed as previously described [10,11], with some modifications. Briefly, GGTase (250 ng) and FTase (20 ng) were incubated in 50 mM Tris–HCl, pH 7.7, 25 μM $ZnCl_2$, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.5 mM Zwittergent 3-12, in the presence of different concentrations of inhibitors in a final volume of 50 μl. The reactions were incubated at 30 °C for 30 min with recombinant H-Ras-CVLL (2.5 μM) and [^{3}H]GGdP (0.1 μM) for GGTase I, and recombinant H-Ras-CVLS (2.5 μM) and [^{3}H]FdP (0.6 μM) for FTase. After incubation the reaction was stopped and filtered on glass fibre filters to separate free from incorporated label. The activity of the inhibitors is reported in Table 1 as their IC_{50}, the concentration at which GGTase and FTase activity was inhibited by 50%.

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