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Synthesis and biological activity of pseudopeptides inhibitors of Ras farnesyl transferase containing unconventional amino acids☆

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Abstract

A study was performed on the structure–activity relationships of a series of phenol derivatives, CVFM analogs, derived from the two most active compounds of a first series ($\mathbf{1_A}$ and $\mathbf{1_B}$) of inhibitors of Ras farnesyl transferase (FTase) that we have recently described. We report the synthesis and the activity of a second series of compounds in which the phenylalanine residue was replaced by unconventional aromatic and non-aromatic amino acids, with varying electronic, lipophilic, steric and conformational properties. The compounds showed to be significantly less active than reference compounds against FT, with the only exception of derivative $\mathbf{3_A}$ ($IC_{50} = 3$ μM), which is slightly more active than $\mathbf{1_A}$ but not $\mathbf{1_B}$. Subsequently we tested the effects of compounds $\mathbf{1_A}$, $\mathbf{1_B}$ and $\mathbf{3_A}$, $\mathbf{3_B}$ on the anchorage-dependent growth of two epithelial cell lines of rats, FRTL-5 and the same line v-Ha-ras transformed. Compound $\mathbf{3_A}$ derived from lead compound $\mathbf{1_A}$, showed an appreciable selectivity against transformed cells. In contrast, compounds derived from derivative $\mathbf{1_B}$ had only a modest cellular activity.

Keywords: Ras farnesyltransferase inhibitor; Phenol derivative; Cellular in vitro activity

1. Introduction

Ras genes are found activated in about 30% of all human neoplasms [2,3]. The Ras proteins (H, N, K-4A and K-4B) are synthesized as cytosolic precursors and localized on the plasma membrane after post-translational modifications [4]. The critical modification required for attachment to the inner membrane and for cell-transforming activity is farnesylation of Ras [5]. This prenylation is catalyzed by the enzyme, farnesylprotein transferase (FTase). Therefore, inhibitors of FTase could act as anticancer agents blocking an essential step for *Ras* activation. FTase uses farnesyl pyrophosphate as a donor and attaches a 15-carbon isoprenoid moiety to the cysteine residue at the fourth position from the COOH-terminus of Ras protein. The cysteine belongs to the terminal tetrapeptide sequence CAAX, where A stands for an aliphatic residue and X stands for methionine or serine [6]. This motif, besides Ras proteins, is shared by nuclear lamins A and B, skeletal muscle phosphorylase kinase, and three retinal proteins that are all substrates for FTase [7]. Since the peptide sequence requirement for the substrates of farnesyl transferase have been elucidated, in the past few years intense efforts of several research groups have been dedicated to the development of numerous highly potent peptidic and nonpeptidic inhibitors of this enzyme [8,9]. As an example the tetrapeptide CVFM is a potent FTase inhibitor ($IC_{50} = 0.06$ μM), which is not farnesylated by the enzyme [10].

Many CAAX peptides and peptidomimetics that inhibit FTase have been described [11], although a preponderance of these compounds contain a terminal cysteine residue as a requirement for good inhibitory activity. Since concerns over thiol-dependent toxicity

☆ Symbols and abbreviations are in accord with recommendations from Ref. [1].

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render these undesirable, we and others have investigated the replacement of the cysteine residue by imidazole [12], pyroglutamine [13], and phenolic rings [14,15] with modest success. As reported above, we prepared a first series of CVFM analogs (Fig. 1) in which the thiol group was replaced by several hydroxybenzoic acids opportunely substituted.

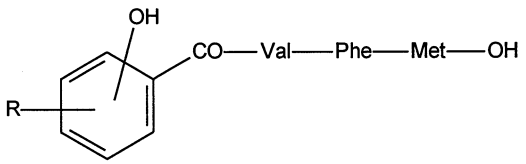
The synthesis, the inhibitory activity against FTase, as well as the cellular activity, of this first series of pseudopeptides has been recently reported [15]. In an effort to further develop structure–activity relationships, in this paper we describe the synthesis and the biological activity of a second series of compounds (Fig. 2) derived from the most active compounds (**1_A** and **1_B**) of the first series in which the phenylalanine residue was replaced by unconventional aromatic and non-aromatic amino acids, with varying electronic, lipophilic, steric and conformational properties. This type of strategy has also been recently employed by others [16,17].

We have chosen to change the amino acid phenylalanine in the most active compounds of the first series, because it has been shown that the A_2 position in the CA_1A_2X motif is a major determinant for nonsubstrate tetrapeptide inhibitor activity [10].

The unusual amino acids that were taken in consideration are 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (**Tic**), 3-(1-naphthyl)alanine (**Nap**), phenylglicine (**Phg**), thienylalanine (**Thi**), (*S*,*S*,*S*)-2-azabiciclo [3.3.0]octane-3-carboxylic (**Aoc**) and (3a*S*,7a*S*)-octahydroindole-2-carboxylic acid (**Oic**), (Fig. 2).

The purpose of our work was also to improve the pharmacological properties of two phenol derivative inhibitors of Ras farnesyl transferase possessing the cellular in vitro activity previously reported by us [15] (Fig. 1).

More active compounds **3_A** and **3_B** were tested in vitro for their FTase inhibitory activity as well as the cellular activity. We also report the growth inhibitory activity of compounds showing an $IC_{50} < 20$ μM against normal and transformed FRTL-5 cells. The synthesized compounds were compared for their activity with compounds **1_A** and **1_B**, taken as reference drugs (see Table 2).



R = H, CH_3, OCH_3, Cl, Br, NO_2

Fig. 1.

2. Chemistry

Oic and Aoc were prepared according to the procedure of Vincent et al. [18] and Teetz et al. [19], respectively, as described in the literature, while Phg, Thi, Tic and Nap were commercially available.

The tripeptides were synthesized by the conventional method in solution using the combination of t-butyloxycarbonyl (Boc) and fluorenylmethyloxy-carbonyl (Fmoc) protecting groups as illustrated in Scheme 1.

Final compounds were obtained by condensation of the appropriate hydroxybenzoic acid derivative with $H–VX_{aa}M–OCH_3$ as reported in Scheme 2.

The starting $Fmoc–X_{aa}M–OCH_3$ were produced from $Fmoc–X_{aa}–OH$ and the methionine methyl ester by the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBT) methods [20].

The Boc-protected tripeptides were synthesized from $Fmoc–X_{aa}M–OCH_3$ derivatives according to the conventional way, which comprises a cycle of deprotection of the Fmoc group with diethylamine (DIEA) in tetrahydrofuran 33% and the subsequent coupling process with Boc–Val–OH by 1-ethyl-3-[3-(*N*,*N*-dimethylamino)-propyl]carbodiimide (WSCD) and 1-hydroxybenzotriazole (HOBT) methods [21,22]. The introduction of non-natural amino acids (Tic, Nap, Phg, Thi, Aoc and Oic), did not require exceptional coupling procedures, although activation with TBTU method [20] was preferred to DCC–HOBT.

The Boc protecting group of the protected tripeptide was removed with 4N hydrochloric acid in dioxane, purified by crystallization and characterized by FAB mass spectroscopy.

The final compounds were obtained from the corresponding deprotected tripeptides by coupling with the appropriate hydroxybenzoic acid derivative by WSCD/HOBT method. The resulted compounds were characterized and converted to their carboxylic form by saponification with 1 N NaOH, successively purified by reverse-phase high performance liquid chromatography (RP-HPLC) to greater than 98% purity and characterized by FAB mass spectroscopy. The reference compound was synthesized according the literature [15]. In Table 1 the analytical data of the final compounds are reported.

3. Experimental

3.1. Chemistry

Capillary melting points were determined on a Büchi SPM-20 apparatus and are reported uncorrected. Optical rotations were recorded on a Atago polax-D polar-

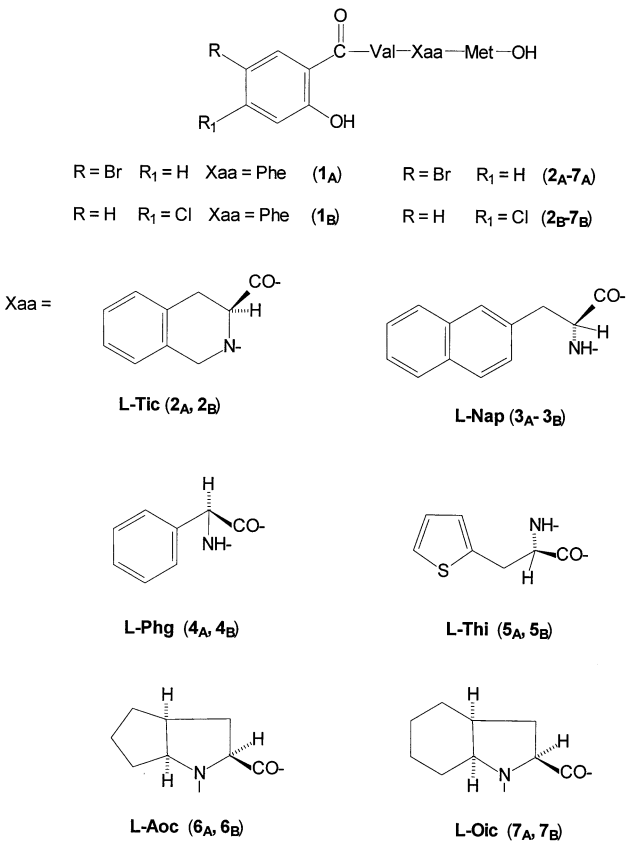
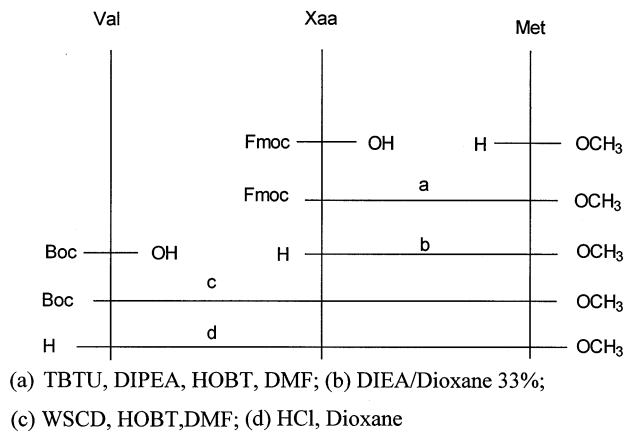


Fig. 2.



Scheme 1. The synthesis of H–$VX_{aa}M$–OCH_3 tripeptides X_{aa} = Tic, Nap, Phg, Thi, Aoc, Oic.

Table 1
Physical constants of the investigated compounds

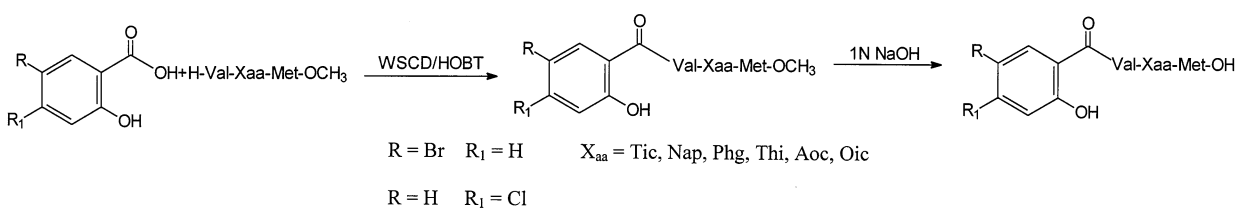
Comp.	FAB MS m/z		HPLC [a,b] t_r (min)	Yield (%) [c]
	Calculated	Found		
$\mathbf{2_A}$	606.62	606.40	10.3	24.5
$\mathbf{3_A}$	644.65	644.50	12.2	21.8
$\mathbf{4_A}$	580.60	580.30	9.5	20.3
$\mathbf{5_A}$	600.63	600.45	10.7	22.5
$\mathbf{6_A}$	584.62	584.42	9.9	21.0
$\mathbf{7_A}$	599.64	599.35	10.5	19.5
$\mathbf{2_B}$	562.12	562.10	10.1	25.0
$\mathbf{3_B}$	600.15	600.00	11.9	20.5
$\mathbf{4_B}$	536.10	536.15	9.2	20.6
$\mathbf{5_B}$	556.13	556.10	10.4	21.7
$\mathbf{6_B}$	540.12	540.00	9.6	20.2
$\mathbf{7_B}$	555.14	555.20	10.2	20.0

[a] Eluents: A (0.1% TFA in CH_3CN) and B (0.1% TFA in H_2O); analytical HPLC on a μ-Bondapak C_{18} silica column (125 Å, 15–20 mm, 30 × 300 mm); linear gradient from 5% A–95% B to 80% A–20% B over 25 min, UV detection at 220 nm, flow rate 1 ml min^{-1}.

[b] The final HPLC purity of the peptides was always > 98%.

[c] Yields of purified peptides were calculated as percentage of the theoretical yield, based on the starting material.

imeter. Elemental analyses were performed on a Perkin–Elmer 2400 CHN analyzer. Analytical results were within ± 0.4% of the theoretical values unless otherwise noted. Precoated plates (E. Merck F 254 silica gel) were used for ascending TLC in the following solvent systems: A, 10:1 $CHCl_3$–MeOH; B, 20:1 $CHCl_3$–MeOH; C, 8:1:1 $CHCl_3$–MeOH–AcOH. The products on thin layer chromatography plates were detected by UV light and either chlorination followed by a solution of 1:1 1% starch–1% KI (v/v) or ninhydrin. For column chromatography silica gel 60 (20–230 mesh ASTM, Merck) was used. Extraction solvents were dried over magnesium sulfate. Solvents used for reactions were dried over 3 Å molecular sieves. Reversed-phase was routinely performed on a Waters Delta-Prep 4000 system equipped with a Waters 484 multiwavelength detector on a μ-Bondapak C-18 silica (125 Å, 15–20 mm, 30 × 300 mm) high performance liquid chromatography (HPLC) column. The opera-



Scheme 2. Synthetic procedure of considered compounds.

tional flow rate was 30 ml min^{-1}. Homogeneity and retention times (t_R) of the purified products were assessed by analytical RP-HPLC with μ-Bondapak C_{18}-125 Å column 10 μm, 3.9 × 300 mm, spherical, with the following solvent system: A 0.1% trifluoroacetic acid (TFA) in CH_3CN, B 0.1% TFA in H_2O, linear gradient 5% A–95% B to 80%A–20% B over 30 min, UV detection at 220 nm, flow rate 1 ml min^{-1}. The final HPLC purity of the peptides was always >98%. The yields of purified peptides were calculated as a percentage of the theoretical yield, based on the starting material. All solvents were filtered and degassed prior to use. Reagent grade material were purchased from Novabiochem and from Aldrich Chemical and were used without further purification. Molecular weights of pseudopeptides were determined by fast atom bombardment (FAB) mass spectrometry on a ZAB 2 SE-Fisons. Dimethylformamide (DMF) was distilled immediately before use over CaH_2.

3.2. General procedure for compounds 1_A–7_A and 1_B–7_B

The main procedures used for the preparation of the protected peptides reported in Schemes 1 and 2 varied little in the individual steps and are therefore summarized in the following general form.

3.2.1. WSCD/HOBT method

A solution of Boc–amino acid (1.0 mmol), amine component (1.0 mmol), and HOBT (1.1 mmol) in CH_2Cl_2 (10 ml) was ice cooled. To this solution was added WSCD·HCl (1.1 mmol). The resulting solution was stirred at this temperature for 2 h and overnight at room temperature and then concentrated, diluted with water, and extracted with EtOAc. The organic layer was washed successively with $NaHCO_3$ solution, water, 0.5 N HCl, and brine and evaporated under reduced pressure. The obtained residue was chromatographed on silica gel eluting with 9:1 $CHCl_3$–MeOH (v/v) and then crystallized from EtOAc–diisopropylether.

3.2.2. TBTU/HOBT method

To a DMF mixture of 1 mmol of Fmoc–X_{aa}–OH, 1.1 mmol of HOBT, 1.1 mmol of TBTU, and 1 mmol of H–Met–OCH_3, 2 mmol of diisopropylethylamine was added under cooling to 0°C. After 16 h at room temperature the reaction mixture was separated from the solvent, the crude residue was taken up in ethyl acetate and washed successively three times with citric acid (5%), sodium bicarbonate (5%), and a saturated solution of sodium chloride. The oil residue was then triturated in ether and pentane to obtain a crude solid compound which was further purified by silica gel column chromatography using 9:1 $CHCl_3$–MeOH (v/v) as eluent.

3.3. Deprotection procedures

The procedures used for the removal of peptide protecting groups are summarized in the following general form.

3.3.1. HCl/dioxane deprotection

To a solution of 1 mmol of peptide and anisole (4 ml) in CH_2Cl_2 (15 ml) was added 4 N HCl (15 ml) in dioxane under ice cooling. The resulting mixture was stirred at this temperature for 15 min and at room temperature for 1 h. The solvent was evaporated under reduced pressure and the residue was crystallized from diethylether. The crystalline product was filtered and dried.

3.3.2. Saponification

Methyl ester groups were removed by treating 1 mmol of peptide in MeOH (8 ml) with 1.2 equivalents 1 N NaOH for 6–8 h at room temperature. The solution was then diluted with water, concentrated in vacuo to remove the methanol, and washed with AcOEt. After cooling at 0°C, the aqueous solution was acidified with 1 N HCl and the product extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated; the residue was purified by preparative RP-HPLC.

3.4. Pharmacology

The more active compounds $\mathbf{3_A}$ and $\mathbf{3_B}$ were tested in vitro for their FTase inhibitory activity as well as the cellular activity [23–25].

3.4.1. Farnesyltransferase protein

The enzyme was a partially purified fraction from a bovine brain homogenate, prepared essentially as described by Reiss et al. [23].

3.5. Farnesyltransferase inhibition assay

The assay that we used, based on the scintillation proximity principle, was the Amersham's Farnesyl Transferase kit. A human lamin-B carboxy terminus sequence peptide (biotin-YRASNRSCAIM) is ^{3}H-farnesylated at the cysteine residue when processed by farnesyl transferase. The resultant complex is captured by a streptavidin-linked SPA beads. In a 100 μl final reaction volume, the test compound was added in 10 μl of DMSO to a 70 μl reaction mixture containing 20 μl of 1:20 diluted [^{3}H]farnesyl pyrophosphate (0.2 μCi), 46 μl of assay buffer (50 mM HEPES, 30 mM $MgCl_2$, 20 mM KCl, 5 mM DTT and 0.01% Triton X-100) and 20 μl of 0.5 mM Biotin–Lamin B peptide in a buffer at pH 7.5 (50 mM HEPES, 25 mM Na_2HPO_4, 20 mM KCl, 5 mM DTT and 0.01% Triton X-100). This mixture was allowed to stand at 37°C for 5 min. The reaction was

started by adding 4 μl of diluted enzyme (~ 2 μg of protein) to the mixture that was incubated at 37 °C for 1 hour. To stop the reaction 150 μl of the stop/bead reagent were added. The samples were counted in a Beckman LS 1801 scintillation counter. Every test compound was assayed at least twice. IC_{50} estimations were made from percent control versus log drug concentration plots.

3.5.1. Cell lines

FRTL-5 is a continuous line of differentiated epithelial cells derived from normal Fisher rat thyroids and represents one of the few available models to study the mechanism of thyroid cell transformation by cellular oncogenes in vitro. These cells have retained the typical markers of thyroid differentiation: thyroglobulin (TG), thyroperoxidase (TPO) and thyrotropin receptor (TSH-R) and depend for growth upon the continuous presence of thyroid stimulating hormone (TSH) in the medium. In vitro experiments on FRTL-5 rat thyroid cells have shown that this oncogene is able, in a single step, of malignantly transforming this cell line [24]. V-Ha-ras transformation results in changes in cellular morphology, an increase in anchorage-dependent growth, a modification of the specific differentiated cell properties and the ability to form tumors when transplanted into syngenic rats.

The FRTL-5 cells were grown in Coon's modified Ham F12 medium supplemented with 5% calf serum and a six hormone mixture: TSH 1×10^{-10} M, insulin 10 mg ml^{-1}, somatostatin 10 ng ml^{-1}, glycyl-L-histidyl-L-lysine acetate 10 ng ml^{-1}, hydrocortisone 1×10^{-8} M and transferrin 5 mg ml^{-1}. FRTL-5 cells fully transformed by the v-Ha-ras oncogene were grown in Coon's modified Ham F12 medium supplemented with 5% calf serum.

3.5.2. Colorimetric cytotoxicity assay

The SRB assay was performed essentially as described by Skehan et al. [25]. Briefly, cells seeded at 1000 cells/well (day 0) were grown in 96-well microtiter plates. Inhibitors were added at 100 μM concentration 24 h after seeding and twice/weekly thereafter. At day 8 the cultures were fixed with trichloroacetic acid and stained for 30 min with 0.4% (w/v) sulforhodamine B (SRB), dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid and protein-bound dye was extracted with 10 mM buffered Tris base for determination of optical density in a computer-interfaced, 96-well microtiter plate reader.

4. Results and discussion

The compounds prepared in this study were tested for their in vitro FTase inhibitory activity as well as the cellular activity.

The Phe residue in the two most active pseudopeptide analogs ($\mathbf{1_A}$ and $\mathbf{1_B}$) of the first series of compounds was substituted with various conformationally-constrained uncoded amino acids also with the purpose of overcoming the bioavailability problem caused by the action of serum proteases that rapidly cleave conventional amino acids. The pseudopeptides prepared in this new series($\mathbf{2_A}$–$\mathbf{7_A}$ and $\mathbf{2_B}$–$\mathbf{7_B}$) showed to be significantly less active than compounds $\mathbf{1_A}$ and $\mathbf{1_B}$ ($IC_{50} = 7$ and 1 μM, respectively), against FT, with the only exception of derivative $\mathbf{3_A}$ ($IC_{50} = 3$ μM), which is slightly more active than $\mathbf{1_A}$ but not $\mathbf{1_B}$. For the other compounds the IC_{50} values were higher than 20 μM except derivative $\mathbf{3_B}$, which showed an IC_{50} of 16 μM. In contrast to our results, others have found that Tic is a suitable replacement for Phe in CA_1A_2X-based FT-inhibitors [26,27]. This observation demonstrates that alkylation of the amine at the A_2 position in our compounds is detrimental to inhibitory potency probably because the aromatic or non-aromatic ring is less able to orient in a favorable position except for naphthyl ring.

The growth inhibition properties of compounds $\mathbf{1_A}$, $\mathbf{1_B}$ and $\mathbf{3_A}$, $\mathbf{3_B}$ against normal and transformed FRTL-5 cells are shown in Table 2. Compound $\mathbf{3_A}$ derived from lead compound $\mathbf{1_A}$ showed an appreciable selectivity against transformed cells. On the contrary compounds derived from the chloro derivative $\mathbf{1_B}$ had only a modest cellular activity. The IC_{50} was not strictly predictive of the cytotoxic activity of these compounds, however inactive compounds with an IC_{50} higher than 100 μM were neither toxic nor selective (results not shown).

The availability in our laboratory of normal rat thyroid epithelial cell lines as well as Ha-ras trans-

Table 2
Cellular activities against normal and transformed cells

Comp.	Cell growth (% control) FRTL-5	FRTL-5 Ha-ras	Selectivity index [a]
$\mathbf{1_A}$	68	69	1
$\mathbf{1_B}$	56	66	0.8
$\mathbf{3_A}$	100	37	2.7
$\mathbf{3_B}$	68.7	75.5	0.9

[a] Selectivity index: growth inhibition of transformed cells/growth inhibition of normal cells.

formed should allow us to discriminate compounds active selectively against malignant cells, that is the final purpose of this kind of studies.

The effort to discovering a better FT inhibitor is continuing in our laboratories and the results will be presented in a forthcoming paper.

Acknowledgements

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