Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 16, pp. 1779-1784, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00314-2

CONSTRAINED ANALOGS OF KCVFM WITH IMPROVED INHIBITORY PROPERTIES AGAINST FARNESYL TRANSFERASE

François-Frédéric Clerc*, Jean-Dominique Guitton*, Nadine Fromage, Yves Lelièvre, Marc Duchesne, Bruno Tocqué, Evelyne James-Surcouf, Alain Commerçon and Jérôme Becquart,

Rhône-Poulenc Rorer S.A.- Centre de Recherches de Vitry-Alfortville
13, Quai Jules Guesde - BP14 - 94403 Vitry-sur-Seine (France)

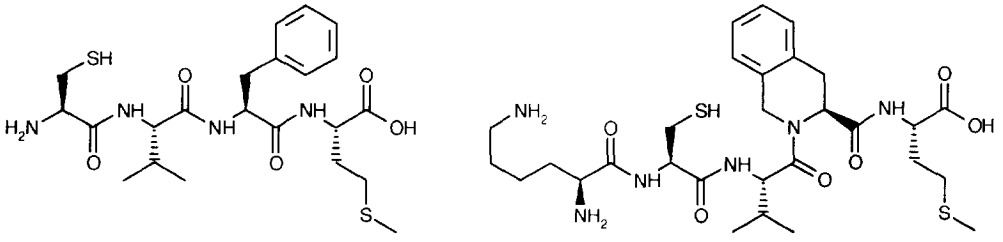
Abstract: Constrained analogs of KCVFM, reported thus far as one of the most active peptidic inhibitors of farnesyl transferase, have been synthesized. Replacement of Val-Phe with Val-Tic and (N-Me)Val-Tic led to dramatically more active analogs possessing favored extended conformations. Based on molecular modelling studies the design and synthesis of various conformational probes to be substituted for Val and Phe led to a good correlation between the ratio of extended conformers and biological activity.

Ras proteins, referred to as H-Ras, N-Ras, K-RasA and K-RasB, are involved in cellular growth regulation in mammalian cells. Oncogenic versions of these proteins have been identified in various important human cancers such as in pancreatic and colon carcinomas[1]. Oncogenic Ras mutations differ from the protooncogenic form found in normal cells by loss of regulatory and catalytic function[2]. It is also known that the Ras proteins must be post-translationally modified in order to retain their full function. This requires anchorage to the cellular membrane by means of a farnesyl group attached to a cysteine located near the protein carboxy-terminus[3]. Furthermore, genetic studies have shown that inhibition of Ras farnesylation blocks Ras-induced cell transformation. These observations raised the possibility that inhibition of Ras farnesylation might slow the growth of cancers in which oncogenic Ras proteins play a role[4].

Ras farnesyl transferase (FTase) is the enzyme which catalyses the transfer of the farnesyl group of farnesylpyrophosphate to the sulfur atom of cysteine-186 of Ras proteins which is part of the CA_1A_2X carboxy-terminal sequence (where C is cysteine-186, A_1 and A_2 are usually aliphatic amino acids and X is methionine or serine). Previously published studies revealed that small peptides with the CA_1A_2X sequence are potent competitive inhibitors of FTase[5]. Systematic change of C, A_1, A_2 and X of the CA_1A_2X sequence by other coded amino acids led to the identification of CVFM (**1**, fig 1) as the most potent inhibitor among 42 variants[5b,c]. This peptide blocks FTase activity (IC50 = 25 nM, FTase purified from rat brain) without being itself farnesylated. Peptide **1**, however, was farnesylated when the amino terminus was modified with an acetyl group[6]. Other inhibitors in this class include for instance CVIM, CVLS, CIFM, and N-acetyl-L-penicillamine-VIM. However all these peptides proved to be inactive in cell-based assays. Various ongoing strategies to find more bioavailable inhibitors are based on the identification of peptidomimetic compounds. Results reported thus far indicate that inhibition of Ras post-translational processing both *in vitro* and in cell-based assays is conceivable by using compounds in which either one or two of the internal peptidic bonds have been replaced by carbon peptidomimetic linkers such as aminomethyl[7], oxymethyl[8], aliphatic[9], aromatic[9c,10], or diazepam[11] moieties. The inclusion of such residues in the tetrapeptide can abolish farnesyl group transfer without affecting its inhibitory potential. These efforts have recently led to almost completely non peptidic structures,

retaining only the apparently critical cysteine-type residue[12]. All of these compounds have provided important information regarding the putative bioactive conformation of CA_1A_2X type inhibitors, yet the issue is still surrounded by some controversy[10b,11,12,13]. We report herein our own efforts in this field starting from the CVFM structure. Since an additional lysine amino acid retains full biological activity (KCVFM corresponds to the last five residues of the K-RasB protein with the penultimate isoleucine replaced by a phenylalanine) and confers water solubility which is critical for accurate *in vitro* evaluation, we employed KCA_1A_2M type compounds in which the two internal amino acids A_1 and A_2 were replaced by various coded and uncoded amino acids. One of our initial objectives was to collect information on the most probable bioactive conformation for KCA_1A_2M type inhibitors.

Figure 1 : Structures of peptides 1 and 2



CVFM (**1**) KCVTicM (**2**)

We initially conducted molecular modelling studies on KCVFM (**1**) using molecular dynamics and energy minimization[14]. Our studies led to the conclusion that nearly 67% of the possible conformations of the pentapeptide KCVFM are extended with a CαCys-CαMet distance greater than 7.5Å. Because the CA_1A_2X box is characterized both by terminal specificity and internal variability in Ras proteins, our simplifying approach toward the examination of the spatial requirements for FTase inhibition was the connection of cysteine and methionine residues by constrained amino acid linkers able to induce a minimum number of conformers.

Materials and Methods:

Peptides (table 1, entries a-e,h-m) were assembled on a Wang resin[15] on a 50-μmolar scale using an Perkin-Elmer-ABI model 431-A peptide synthesizer. A Fmoc strategy was chosen for synthesis, the side-chains of lysine and cysteine were protected by the Boc and trityl group respectively. The instrument was operated using constructor's "standard Fmoc" programming cycles with minor modifications. After each coupling step, the Fmoc group was removed by treatment with 20% piperidine in N-methylpyrrolidone-2. For the synthesis of peptide Lys-Cys-NMeVal-Tic-Met (entry l) : Fmoc-Met was coupled as its symmetrical anhydride to the resin, Fmoc-Tic was coupled as its N-hydroxybenzotriazole ester, Fmoc-NMeVal was coupled as its O-dicyclohexylurea (addition of N-hydroxybenzotriazole in the reaction mixture was omitted), Fmoc-Cys(Trt) was double-coupled as its O-dicyclohexylurea and Fmoc-Lys(Boc) was coupled as its N-hydroxybenzotriazole ester. Cleavage from the resin and global deprotection were effected by 1.5 hour treatment in a mixture of trifluoroacetic acid / phenol / ethanedithiol / thioanisole / water (40/3/1/2/2 - v/w/v/v/v). After filtration, the solution was concentrated *in vacuo* and the product recovered by precipitation in *tert*-butylmethylether / petroleum ether (2/1 - v/v), purified by preparative HPLC on a C_{18} 100 Å column (250 x 10 mm provided by BioRad) and freeze dried.

The *in vitro* inhibition assay[16] was carried out on human farnesyl transferase prepared using the method of Reiss et al. with some modifications[17] and was adapted to a TCA precipitation assay in microtiter plates. The most significant results are reported in Table 1.

Results and discussion:

As shown in Table 1, after random replacement of A_1 and A_2, a tremendous improvement in the inhibitory properties was observed with peptide **2** (fig. 1) in which phenylalanine was replaced by (L)-1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (Tic) as the A_2 residue (Table 1, compare entries i and j). Such an improvement has been independently observed by Marsters et al. who reported that the tetrapeptide acetyl-CVTicM was twenty-two fold more active than acetyl-CVFM[11b]. We conducted molecular dynamics studies on KCVTicM but, unlike previously reported data with acetylCVTicM, we found that, using simulated annealing protocol[14], this peptide could adopt two types of conformations. The major family of conformers (about 80%) proved to be extended with an average C2-C5 (CαCys-CαMet) distance close to 10Å while the minor one (10%) adopts a turn-like structure with a C2-C5 (CαCys-CαMet) distance close to 5Å (10% exhibit a bend conformation). Since molecular modelling studies also show that 67% of KCVFM conformers tend to adopt extended conformations (vide supra), we decided to further investigate the possibility of a correlation between the extended conformation of KCA_1A_2M type peptides and their inhibition properties.

Table 1 : Structure, conformation and inhibitory activities of KCA_1A_2M peptides

Entry	Substituent A_1-A_2	% extended structure	FTase inhibition IC50 (μM)
a	Val-(d)Tic	6	100
b	(d)Pro-Tic	10	100
c	Pro-(d)Tic	15	50
d	(d)Val-Tic	20	15
e	(d)Pro-(d)Tic	34	100
f	(N-Me)Val-Phe[c]	55	1
g	Tbg-Phe[c]	56	1
h	(N-Me)Val-Phg	65	1
i	Val-Phe	67	1
j	Val-Tic	80	0.020
k	Pro-Tic	91	2
l	(N-Me)Val-Tic	95	0.005
m	Tbg-Tic	98	0.040

[a] Tbg = tert-butylglycine. [b] Phg = phenylglycine. [c] prepared by another approach[18]

Thus we introduced various other conformationally-constrained amino acids at A_1 and A_2 able to reinforce or diminish the extended character of the pentapeptides. As shown in Table 1 (entries a, c and e), pentapeptides possessing (d)Tic at A_2 exhibit preferred turn-like structures and are weakly active. Substitution of (d)Pro for A_1, when associated with Tic or (d)Tic, give comparable results as highlighted by entries b

and e. On the contrary, the Pro-Tic pair leads mainly to extended conformers and improved inhibition properties (entry k). As emphazed by entries f, g and i, Val, (N-Me)Val and Tbg associated with Phe induce almost equal effects on the conformations ratio and biological activity. Lastly entries j, k, l and m clearly demonstrate the strong influence of the Tic residue on the extended conformation as well as the inhibition potency.

Fig. 2: Representative energy-minimized structure for extended KC(N-Me)VTicM

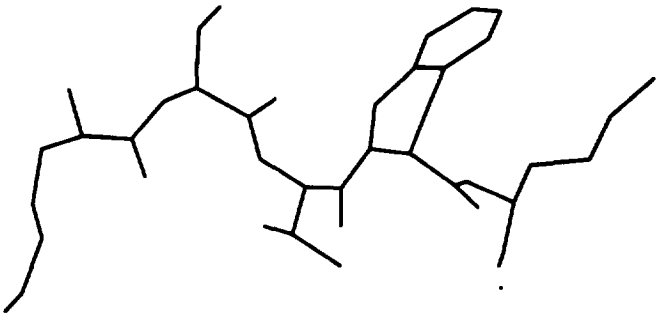
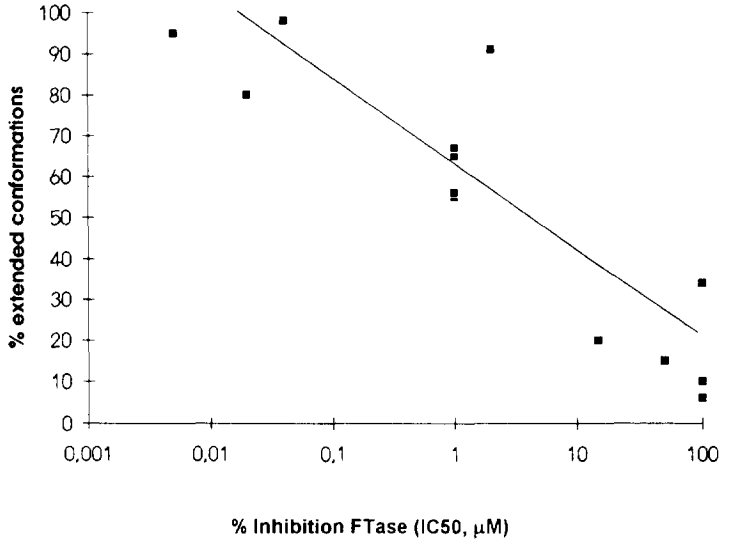


Fig 3: Correlation between the extended conformations and the % of FTase inhibition at 50 μM.



Our results clearly show that, unlike previously reported data in favor of a turn-like backbone structure for the bioactive conformation for the CA_1A_2M type peptides[11,13] and in agreement with the proposition made by Hamilton, Sebti and coworkers[10b,12], the extended conformation looks more preferable for excellent FTase inhibition. Moreover a rather good correlation is observed between the ratio of extended conformations and biological activity (fig. 3). Further results will be shortly published regarding the design of new peptidic and pseudopeptidic FTase inhibitors.

Acknowledgements: We thank Dr. M. Vuilhorgne and coll. for structural analyses, H. Dubois and O. Boniface for assistance in peptides synthesis, R. Boulay for biological evaluation and C. Colonne for FTase purification. We also thank Neosystem S.A. for the synthesis of peptides. The financial support by Bio Avenir is gratefully acknowledged. We are also very indebted to Dr. C.J. Burns for critical reading of the manuscript.

References and notes:

1. Bos, J.L. *Cancer Res.* **1989**, *49*, 4682-4689.
2. Barbacid, M. *Ann. Rev. Biochem.* **1987**, *56*, 779-827.
3. a) Willumsen, B.M.; Norris, K.; Papageorge, A.G.; Hupert, N.L.; Lowy, D.R. *EMBO J.* 1984, *3*, 2581-2585. b) Hancock, J.F.; Magee, A.I.; Childs, J.E.; Marshall, C.J. *Cell* **1989**, *57*, 1167-1177. c) Jackson, J.H.; Cochrane, C.G.; Bourne, J.R.; Solski, P.A.; Buss, J.E.; Der, C. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3042-3046. d) Casey, P.; Solski, P.;Der, C.J.; Buss, J.E. *Proc. Natl. Acad. Sci.* **1989**, *86*, 8323-8327. e) Kato, K.; Cox, A.D.; Hisaka, M.M.; Graham, S.M.; Buss, J.E.; Der, C.J. *Proc. Natl. Acad. Sci.* **1992**, *89*, 6403-6407.
4. For recent reviews on the inhibition of Ras FTase, see: a) Omura, S.; Takeshima, H. *Drugs of the future* **1994**, *19*, 751-755. b) Tamanoi, F. *TIBS* **1993**, *18*, 349-353.
5. a) Reiss, Y.; Goldstein, J.L.; Seabra, M.C.; Casey, P.J.; Brown, M.S. *Cell* **1990**, *62*, 81-88. b) Reiss, Y.; Stradley, S.J.; Gierasch, L.M.; Brown, M.S.; Goldstein, J.L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 732-736. c) Golstein, J.L.; Brown, M.S.; Stradley, S.J.; Reiss, Y.; Gierasch, L.M. *J. Biol. Chem.* **1991**, *266*, 15575-15578. d) Pompliano, D.L.; Rands, E.; Schaber, M.D.; Mosser, S.D.; Anthony, N.J.; Gibbs, J.B. *Biochemistry* **1992**, *31*, 3800-3807.
6. Kohl, N.E.; Mosser, S.D.; Jane deSolms, S.; Giuliani, E.A.; Pompliano, D.L.; Graham, S.L.; Smith, R.L.; Scolnick, E.D.; Oliff, A.; Gibbs, J.B. *Science*, **1993**, *260*, 1934-1937.
7. Brown, M.S.; Goldstein, J.L.; Paris, K.J.; Bumier, J.P.; Marsters, J.C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *89*, 8313-8316.
8. a) Garcia, A.M.; Rowell, C.; Ackermann, K.; Kowalczyk, J.J.; Lewis, M.D. *J. Biol. Chem.* **1993**, *268*, 18415-18418. b) Cox, A.D.; Garcia, A.M.; Westwick, J.K.; Kowalczyk, J.J.; Lewis, M.D.; Brenner, D.A.; Der, C.J. *J. Biol. Chem.* **1994**, *269*, 19203-19206. c) Graham, S.L.; Jane deSolms, S.; Giuliani, E.A.; Kohl, N.E.; Mosser, S.D.; Oliff, A.I.; Pompliano, D.L.; Rands, E.; Breslin, M.J.; Deana, A.A., Garsky, V.M.; Scholz, T.H.; Gibbs, J.B.; Smith, R.L. *J. Med. Chem.* **1994**, *37*, 725-732. d) Leftheris, K.; Kline, T.; Natarajan, S.; DeVirgilio, M.K.; Cho, Y.H.; Pluscec, J.; Ricca, C.; Robinson, S.; Seizinger, B.R.; Manne, V.; Meyers, C.A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 887-892. e) Prendergast, G.C.; Davide, J.P.; Jane deSolms, S.; Giuliani, E.A.; Graham, S.L.; Gibbs, J.B.; Oliff, A.; Kohl, N.E. *Mol. Cell. Biol.* **1994**, *14*, 4193-4202.
9. a) Kohl, N.E.; Wilson, F.R.; Mosser, S.D.; Giuliani, E.; Jane deSolms, S.; Conner, M.W.; Anthony, N.J.; Holtz, W.J.; Gomez, R.P.; Lee, T-J.; Smith, R.L.; Graham, S.L.; Hartman, G.D.; Gibbs, J.B.; Oliff, A. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 9141-9145. b) Wai, J.S.; Bamberger, D.L.; Fisher, T.E.; Graham, S.L.; Smith, R.L.; Gibbs, J.B.; Mosser, S.D.; Oliff, A.I.; Pompliano, D.L.; Rands, E.; Kohl, N.E. *Bioorg. Med. Chem.* **1994**, *2*, 939-947. c) Harrington, E.M.; Kowalczyk, J.J.; Pinnow, S.L.; Ackermann, K.; Garcia, A.M.; Lewis, M.D. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2275-2780.
10. a) Nigam, M.; Seong, C-M.; Qian, Y.; Hamilton, A.D.; Sebti, S.M. *J. Biol. Chem.* **1993**, *268*, 20695-20698. b) Quian, Y.; Blaskovich, M.A.; Saleem, M.; Seong, C.M.; Wathen, S.P.; Hamilton, A.D.; Sebti, S.M. *J. Biol. Chem.* **1994**, *269*, 12410-12413.
11. a) James, G.L.; Goldstein, J.L.; Brown, M.S.; Rawson, T.E.; Somers, T.C.; McDowell, R.S.; Crowley, C.W.; Lucas, B.K.; Lewinson, A.D.; Marsters, J.C. *Science*, **1993**, *260*, 1937-1942. b) Marsters, J.C.; McDowell, R.S.; Reynolds, M.E.; Oare, D.A.; Somers, T.C.; Stanley, M.S.; Rawson, T.E.; Struble, M.E.; Burdick, D.J.; Chan, K.S.; Duarte, C.M.; Paris, K.J.; Tom, J.Y.K.; Wann, D.T.; Xue, Y.; Burnier, J.P. *Bioorg. Med. Chem.* **1994**, *2*, 949-957.
12. Vogt, A.; Quian, Y.; Blaskovich, M.A.; Fossum, R.D.; Hamilton, A.D.; Sebti, S.M. *J. Biol. Chem.* **1995**, *270*, 660-664.
13. Stradley, S.J.; Rizo, J.; Gierasch, L.M. *Biochemistry*, **1993**, *32*, 12586-12590.
14. Molecular modelling studies were performed using the Insight II / Discover package (Biosym Technologies Inc.) on a Silicon Graphics Indigo II workstation. Simulated annealing protocol (900°K (2ps), 300°K (2ps), minimization) was used to generate 100 structures for each modelled peptide. The

simulations were carried out *in vacuo* with N and C termini uncharged to avoid artefactual turn formation. The obtained structures were analyzed using Insight II tools and classified into structural families.

15. Wang, S.S. *J. Am. Chem. Soc.* **1973**, *95*, 1328-1333.
16. This assay allows the measurement of [^{3}H] farnesyl transferred from [^{3}H] farnesyl pyrophosphate (FPP) on the protein and is drawn from the method of Reiss et al.[5a] with some modifications.[^{3}H]FPP is purchased from NEN and p21 H-*ras* is prepared as described by Rey et al.[19]

    The standard reaction mixture contains, in a 1 ml 96 wells microplates (Titer Plate® Beckman), the following concentrations of components in a final volume of 60 µl: 50 mM Tris-chloride (pH 7.5), 5 mM $MgCl_2$, 5mM dithiothreitol (DTT), 0.2% octyl β-D-glucopyranoside, 5 ng (20 µl) of purified farnesyl transferase and either 10 µl of the same buffer or 10 µl of inhibitor at a chosen concentration, are added. Then, after an incubation of 10 minutes at 0°C, 200 pmoles (20 µl) of p21 H-*ras* are added, and the reaction is initiated with 4.5 pmoles (10 µl) of [^{3}H]FPP (61000 dpm/pmoles).
    The incubation is carried out at 37°C for 20 minutes, then is stopped by 0.4 ml of a 30% (W/V) TCA in methanol, then, 0.4 ml of 0.1%SDS (W/V) in methanol are added. After 4 min of agitation, the microplate is maintained at 0°C for 1h on ice.

    The TCA precipitate containing mixture is filtrated on glass fiber (Filtermat® Pharmacia, or GF/C Whatman paper) with a filtration unit (CombiCell Harvester® SKATRON) and washed with 6% TCA (W/V) in water. The filter is dried for 3 minutes in a microwave oven at maximum power, then impregnated with scintillant Meltilex® melt under hot air from a fan up to a relative transparency. Then, the filter is counted in the β Plate® scintillation counter (LKB). Each assay is carried out in triplicate and compared to blanks with or without farnesyl transferase according to either a farnesyl transferase inhibition or a farnesyl transferase activity is measured.
    One Activity Unit being defined as the amount of enzyme which transfers 1 pmole of farnesyl group per hour, determined by the TCA assay method.
17. Farnesyl-protein transferase purification is drawn from the method of Reiss et al.[5a,20] with some modifications. This enzyme is purified from the cytosol of human monocytes THP1 (ATCC TIB 202). Culture cells are washed with ice cold phosphate buffer saline and incubated at 4°C for 30 min in lysis buffer (10 mM HEPES buffer pH 7.4, containing 1mM $MgCl_2$, 1mM EGTA, 1mM PMSF, 0.5 µM Pepstatin, 1µM Leupeptin, 10 mM sodium pyrophosphate and 0.1 mM orthovanadate). The lysate is centrifuged at 100,000g for 15 min at 4°C after addition of NaF (final concentration 100 mM) and the supernatant is further centrifuged at 100,000g for 45 min at 4°C and fractionated by ammonium sulfate. The 30-60% ammonium sulfate fraction is dissolved in 50 mM Tris-HCl buffer pH 7.5 containing 1 mM dithiotreitol, 20 µM $ZnCl_2$, and 0.2% Hecameg, then dialysed for 15h against 4l of fresh buffer and again against 10l of fresh buffer for 20h at 4°C. The dialysate material is applied to a fast flow Q Sepharose column equilibrated with buffer and eluted with a NaCl gradient. Then, the 0.23M fraction is collected and a 3/4 dilution is carried out in the affinity buffer (50 mM Tris pH 7.5, 1mM DTT, 20µM $ZnCl_2$, 0.2% octyl-β-D-glucopyranoside). This sample is applied on a affinity column of TKCVIM-CH Sepharose (made from TKCVIM and activated CH Sepharose as the Pharmacia protocol). Then, the resin is washed with several column volumes of 1M NaCl containing affinity buffer to remove the non specifically bound proteins. Farnesyl transferase is eluted with a 50 mM Tris succinate buffer pH 5.4, 1mM DTT, 20 µM Zn Cl_2, 0.3% octyl-β-D-glucopyranoside, 0.5M NaCl. Since the farnesyl transferase has a poor stability at lower pH, 9.5 ml fractions are collected into 0.5 ml of 1M Tris pH 8. Then, all the activity is eluted in a single protein peak, free of geranylgeranyl transferase activity. This fraction is aliquoted and stored at -80°C.
18. Peptides provided by Neosystem S.A. (Strasbourg, France).
19. Rey, I.; Soubigou, P.; Debussche, L.; David, C.; Morgat, A.; Bost, P-E.; Mayaux, J-F.; Tocqué, B. *Mol. Cell. Biol.* **1989**, *9*, 3904-3910.
20. Reiss, Y.; Seabra, M.C.; Armstrong, S.A.; Slaughter, C.A.; Goldstein, J.L.; Brown, M.S. *J. Biol. Chem.* **1991**, *266*, 10672-10677.

(*Received in Belgium* 14 *April* 1995; *accepted* 26 *June* 1995)