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New Ras *CAAX* mimetics: Design, synthesis, antiproliferative activity, and RAS prenylation inhibition

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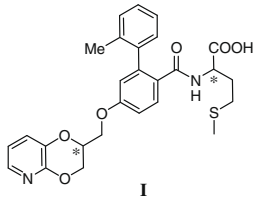
ABSTRACT

Mimetics of the C-terminal *CAAX* tetrapeptide of Ras protein were designed replacing cysteine (*C*) by 2-hydroxymethylbenzodioxane or 2-aminomethylbenzodioxane, respectively etherified and amidified with 2′-methyl or 2′-methoxy substituted 2-carboxy-4-hydroxybiphenyl and 2,4-dicarboxybiphenyl. These pluri-substituted biphenyl systems, used as internal spacer and *AA* dipeptide bioisoster, were linked to the methyl ester of L-methionine, glycine or L-leucine by an amide bond. The resultant twelve pairs of stereoisomers at the dioxane C-2 were tested for antiproliferative effect finding the maximum activity for derivatives with methyleneoxy linker between benzodioxane and 2′-methylbiphenyl. Of these compounds, the one with terminal methionine and *S* configuration proved a good Ras prenylation inhibitor in a cell-based assay.

A vast number of proteins in animals are subject to prenylation, that is the addition of a hydrophobic isoprenoid catalyzed by enzymes such as farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase). This process has proven to be critical for the biological function of several proteins involved in signal transduction, since it strongly influences their association to cellular membrane and protein–protein interactions.[1] Great interest in inhibition of protein prenylation, in particular of Ras proteins farnesylation, arises from the finding that farnesylation is essential for the role of these proteins in mitogenesis and of their mutated forms in oncogenesis. Moreover, proliferation of smooth muscle cells (SMCs) in the arterial wall in response to vascular injury is an important pathogenetic factor of vascular disorders such as atherosclerosis and restenosis after angioplasty.[2] The importance of H-Ras in SMC proliferation in response to vascular injury has been shown by the adenoviral delivery of a dominant negative mutant of H-Ras, which effectively inhibits SMC accumulation after balloon injury of rat carotid artery.[3]

Recently, we have reported that the stereoisomers of 2-*o*-tolyl substituted 4-hydroxybenzamides of methionine, pyridodioxan-2-ylmethyl etherified at the phenolic function, (**I**) have a significant antiproliferative effect on human aortic smooth muscle cells (SMCs) demonstrating that the two stereoisomers of **I** with higher antiproliferative activity (17 and 59 μM IC_{50}) interfere with Ras farnesylation, as indicated by the appearance of unprenylated Ras in the total protein extract of aortic SMCs incubated with **I** stereoisomers under the same conditions utilized for evaluating cell proliferation.[4]



I

Compound **I** and its analogues with methoxyl in lieu of methyl or methyleneamido in place of methyleneoxy linker or with both these variations had been designed using the Ras C-terminal tetrapeptide *CAAX* as a template, since such a tetrapeptide is the minimum length co-substrate accepted by FTase for in vitro reaction.[5–9] Applying this commonly practiced strategy, we had replaced cysteine with pyridodioxane and the internal dipeptide *AA* with 4-hydroxy- or 4-aminocarbonyl-2-biphenylcarboxylic acid. Pyridodioxane had been chosen as rigidified analogue of 3-pyridyloxymethyl, a substructure successfully used to replace cysteine in FTase inhibitors,[9] with improved hydrogen bond acceptor capability and additional presence of a stereocenter with respect to 3-pyridyloxymethyl. Based on the encouraging biological data of **I** and of its analogues,[4] we designed compounds **1**–**24**, whose salient feature is the bioisosteric replacement of pyridodioxane with benzodioxane, a modification we have previously experienced as ameliorating in other classes of biologically active compounds.[10]

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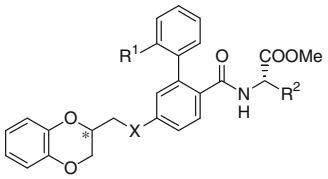


1, **2**	R^1 = Me	R^2 = CH_2CH_2SMe	X = O
3, **4**	= OMe	= CH_2CH_2SMe	X = O
5, **6**	= Me	= H	X = O
7, **8**	= OMe	= H	X = O
9, **10**	= Me	= CH_2CHMe_2	X = O
11, **12**	= OMe	= CH_2CHMe_2	X = O
13, **14**	= Me	= CH_2CH_2SMe	X = NHCO
15, **16**	= OMe	= CH_2CH_2SMe	X = NHCO
17, **18**	= Me	= H	X = NHCO
19, **20**	= OMe	= H	X = NHCO
21, **22**	= Me	= CH_2CHMe_2	X = NHCO
23, **24**	= OMe	= CH_2CHMe_2	X = NHCO

Indeed, 2-substituted 2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine represents only one of the two pyridodioxane systems containing conformationally blocked 3-pyridyloxymethyl substructure, the other being 3-substituted 2,3-dihydro-1,4-dioxino[2,3-*c*]pyridine, which we have not yet evaluated (Fig. 1). However, before undertaking the synthesis of 2,3-dihydro-1,4-dioxino[2,3-*c*]pyridine derivatives, it seemed worthwhile to first pass through the less troublesome bioisosteric replacement of 2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine with benzodioxane thus eliminating the variable of nitrogen position in the aromatic ring of the bicyclic system. Such a simplifying modification could have beneficial effects, we reasoned, in the case of unfavorable position of nitrogen in the 2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine system of **I** and its analogues.

Compounds **1**–**24** were designed on this hypothesis, but also considering alternative aminoacids to terminal methionine such as glycine and leucine, which are characterized by quite different steric bulkiness and lipophilicity. Finally, only in vitro cellular tests being available to us for biological evaluation, we decided to enhance cell permeability esterifying the aminoacid carboxyl.

To synthesize compounds **1**–**12**, characterized by methyleneoxy linker between terminal benzodioxane and biphenyl core, we firstly prepared the two non-aminoacidic building blocks, namely 2-mesyloxymethylbenzodioxane **25**, in both the enantiomeric forms, and methyl 4-hydroxybenzoate, *o*-tolyl or *o*-anisyl substituted at position 2 (**32** and **33**, respectively). The synthesis of (*S*)- and (*R*)-**25** was accomplished according to procedures we had previously reported,[11] while **32** and **33** were prepared from 2-bromo-4-nitrobenzoic acid by a sequence of reactions identical to that employed for the corresponding ethyl esters (Scheme 1).[12–18]

Nucleophilic displacement of mesylate group of (*R*)-**25** by **32** and **33** yielded (*S*)-**34** and (*S*)-**35**, respectively.[19,20] Both these methyl esters were hydrolyzed and the resultant carboxylic acids (*S*)-**36**[21] and (*S*)-**37**[22] condensed with L-methionine or L-leucine or glycine methyl

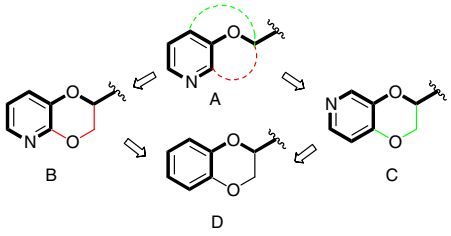
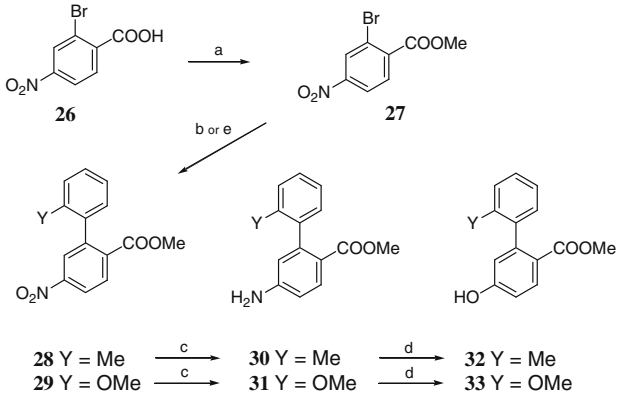


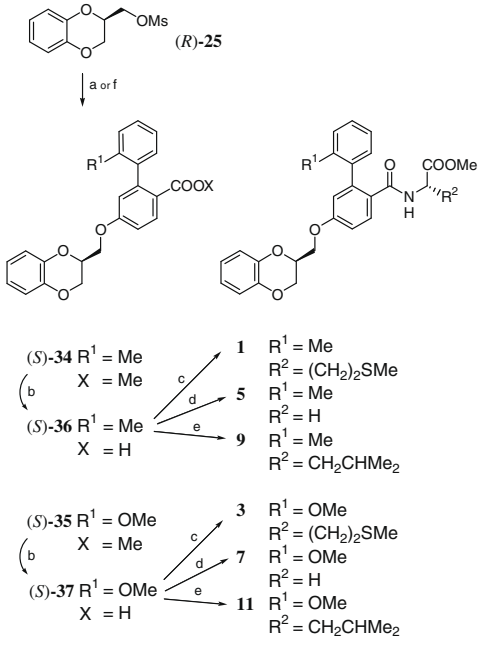
Figure 1. 2-Substituted 2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine (B), 3-substituted 2,3-dihydro-1,4-dioxino[2,3-*c*]pyridine (C) as rigid systems containing 3-pyridyloxymethyl substructure (A) and 1,4-benzodioxane (D) as bioisoster of both.



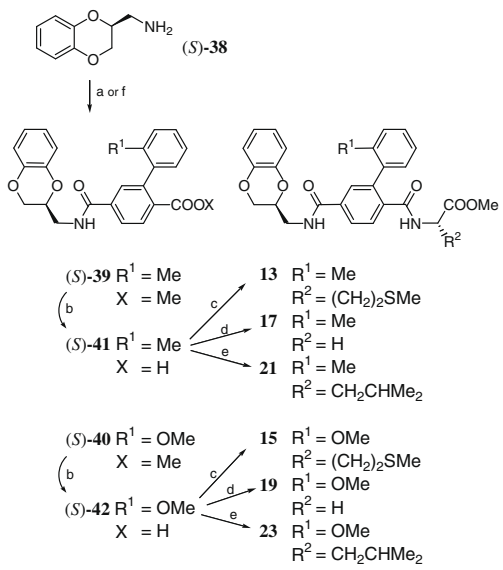
Scheme 1. Synthesis of the methyl esters of 2-*o*-tolyl- and 2-*o*-anisyl-4-hydroxybenzoic acid: (a) methanol, methyl orthoformate, H_2SO_4 conc., reflux, 12 h, 87%; (b) tolyl boronic acid, $Pd(PPh_3)_4$, 2 M Na_2CO_3, ethanol, toluene, reflux, 12 h, 86%; (c) H_2-Pd/C, methanol, 3 h, 83% (**30**) and 64% (**31**); (d) $NaNO_2$, 2 N H_2SO_4, acetone, −10 °C and then room temperature, 12 h, 84% (**32**) and 85% (**33**); (e) *o*-methoxyphenyl boronic acid, $Pd(PPh_3)_4$, 2 M Na_2CO_3, ethanol, toluene, reflux, 12 h, 82%.

ester to give the target compounds **1**, **3**, **5**, **7**, **9** and **11** with *S* configuration at the dioxane stereocenter (Scheme 2).[23–28] The corresponding stereoisomers with *R* configuration at the dioxane stereocenter, namely the target compounds **2**, **4**, **6**, **8**, **10** and **12**, were synthesized from (*S*)-**25** and L-methionine or L-leucine or glycine methyl ester by the same sequence of reactions illustrated in Scheme 2.[29–38]

The synthesis of compounds **13**–**24**, characterized by methyleneamido linker between benzodioxane and biphenyl, required unichiral aminomethylbenzodioxane **38** and methyl 2-*o*-tolyl-4-carboxybenzoate or its *o*-anisyl analogue. The two methyl benzoates and the enantiomers of **38** were prepared according to previously reported procedures.[4,39,40] To prepare **13**, **17** and **21**,



Scheme 2. Synthesis of compounds **1**, **5**, **9**, **3**, **7** and **11**: (a) **32**, NaH, DME, −20 °C and then reflux, 72 h, 69%; (b) 3 N KOH, acetone, 50 °C, 12 h, 80% ((*S*)-**36**) and 76% ((*S*)-**37**); (c) L-methionine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 90% (**1**) and 94% (**3**); (d) glycine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 88% (**5**) and 69% (**7**); (e) L-leucine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 90% (**9**) and 85% (**11**); (f) **33**, NaH, DME, −20 °C and then reflux, 72 h, 74%.



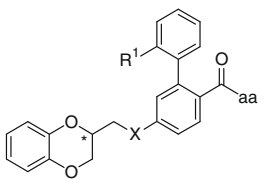
Scheme 3. Synthesis of compounds **13**, **17**, **21**, **15**, **19** and **23**: (a) methyl 2-*o*-tolyl-4-carboxybenzoate, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 12 h, room temperature, 80%; (b) 3 N KOH, methanol, reflux, 12 h, 95% ((*S*)-**41**) and 96% ((*S*)-**42**); (c) L-methionine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 68% (**13**) and 78% (**15**); (d) glycine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 63% (**17**) and 62% (**19**); (e) L-leucine methyl ester hydrochloride, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 24 h, room temperature, 84% (**21**) and 70% (**23**); (f) methyl 2-*o*-anisyl-4-carboxybenzoate, HOBt, EDAC, DMF, 15 min, room temperature and then TEA, 12 h, room temperature, 81%.

methyl 2-*o*-tolyl-4-carboxybenzoate was reacted with (*S*)-**38** obtaining (*S*)-**39**.[41] Successive hydrolysis of methyl ester yielded the carboxylic acid (*S*)-**41**,[42] which was condensed with L-methionine or glycine or L-leucine methyl ester to give the target compounds **13**, **17** and **21** with *S* configuration at the dioxane stereocenter.[43–45] Their methoxy analogues **15**, **19** and **23** were obtained by the same synthetic route, using methyl-2-*o*-anisyl-4-carboxybenzoate as a biphenyl synthon (Scheme 3).[46–50] The corresponding stereoisomers with *R* configuration at the dioxane stereocenter, namely the target compounds **14**, **16**, **18**, **20**, **22** and **24**, were synthesized from (*S*)-**38** and L-methionine or L-leucine or glycine methyl ester by the same sequence of reactions illustrated in Scheme 3.[51–60]

Compounds **1**–**24** were tested in a cellular assay that measured inhibition of human aortic SMC proliferation.[61] As listed in the Table 1, the IC_{50} values ranged from submicromolar to submillimolar, most of compounds showing 1–100 μM IC_{50}. The twelve compounds with oxymethylene linker, the 'ethers', are all more active than those with methyleneamido linker, the 'amides'. Moreover, out of the 'ethers', but not out of the less potent 'amides', the methyl substituted compounds are invariably more potent than the corresponding methoxy substituted analogues. Such a result is consistent with what we have reported for the pyridodioxane analogues,[4] whose most active derivatives also present the biphenyl core methyl substituted and linked to dioxane through oxymethylene bridge. On the contrary, coherent activity trends cannot be recognized with reference both to the configuration of the benzodioxane stereocenter, as previously found for the pyridodioxane stereocenter, and to terminal aminoacid residue. Rather surprisingly, methionine, one of the typical terminal amino acid of *CAAX* motif, is not associated with the highest activities excepting **1** and more than one leucine or glycine derivative shows lower IC_{50} values. However, it is significant what resulted when the effect on protein prenylation was evaluated for the compounds with the highest antiproliferative activity, namely **1**, **5**, **6**, **7**, **8**, **9**, **10**, **11** and **12**, by Western blot analysis of Ras from a total protein extract of cells incubated under the same experimental conditions utilized for evaluating cell proliferation.[62] Only the methionine derivative **1** proved to directly interfere with Ras prenylation (IC_{50} 2.8 μM), as indicated by the accumulation of unprenylated Ras into the cell (Fig. 2);[63] the other compounds, with terminal glycine (**5**–**8**) or leucine (**9**–**12**), had no effect on Ras prenylation, including the most antiproliferative compound **6**, or showed much lower prenylation inhibition than **1**.[64]

The final question is whether the replacement of 2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine by benzodioxane is productive. Appropriate comparison can be made only on the basis of antiproliferative activity, determined for both the classes of compounds, benzodioxanes and pyridodioxanes, and considering the methyl esters, rather than the free carboxylic acids, since the common biological test was an in vitro cellular assay. Therefore, we prepared the methyl ester of the *SS* isomer of **I**, that is, the pyridodioxane analogue of **1**. The *SS* isomer of **I** (free acid) had previously proved to exert antiproliferative effect with 158 μM IC_{50}. We found that conversion into methyl ester increases antiproliferative activity lowering IC_{50} from 158 to 30 μM and produces direct interference with Ras prenylation at 50 μM. Considering that 6.6 μM and 2.8 μM concentrations of **1** are required for 50% inihibition of cellular proliferation and of Ras prenylation respectively (Fig. 2), affirmative answer to the above question seems reasonable. Furthermore, in the case of previous pyridodioxanes, FTase inhibition had been determined and preliminary results, obtained by using a specific antibody for unprenylated Rap1, a GGTase I substrate, indicated that such inhibition was FTase specific. Though further analyses will be necessary on both the series

Table 1
Antiproliferative effect of compounds **1**–**24** on human aortic SMCs



X	Terminal aminoacid methyl ester (aa)	R^1 = Me (*S* tolyl derivs)	IC_{50} (μM)	R^1 = Me (*R* tolyl derivs)	IC_{50} (μM)	R^1 = OMe (*S* anisyl derivs)	IC_{50} (μM)	R^1 = OMe (*R* anisyl derivs)	IC_{50} (μM)
O	L-Met	1	6.6 ± 3.8	2	193 ± 61.4	3	230 ± 24.5	4	204 ± 76.7
	L-Gly	5	27 ± 8.2	6	0.7 ± 0.4	7	71 ± 3.4	8	35 ± 12.6
	L-Leu	9	3.0 ± 2.3	10	5.2 ± 4.3	11	32 ± 12.5	12	17 ± 9.9
NHCO	L-Met	13	87 ± 42.3	14	533 ± 89.3	15	252 ± 65.3	16	647 ± 89.5
	L-Gly	17	113 ± 59.7	18	89 ± 26.6	19	122 ± 29.2	20	76 ± 43.7
	L-Leu	21	103 ± 23.1	22	88 ± 21.5	23	185 ± 35.6	24	469 ± 89.0

The IC_{50} (μM) values were determined by linear regression analysis of the logarithm of the concentration versus the percentage of the inhibitory effect.

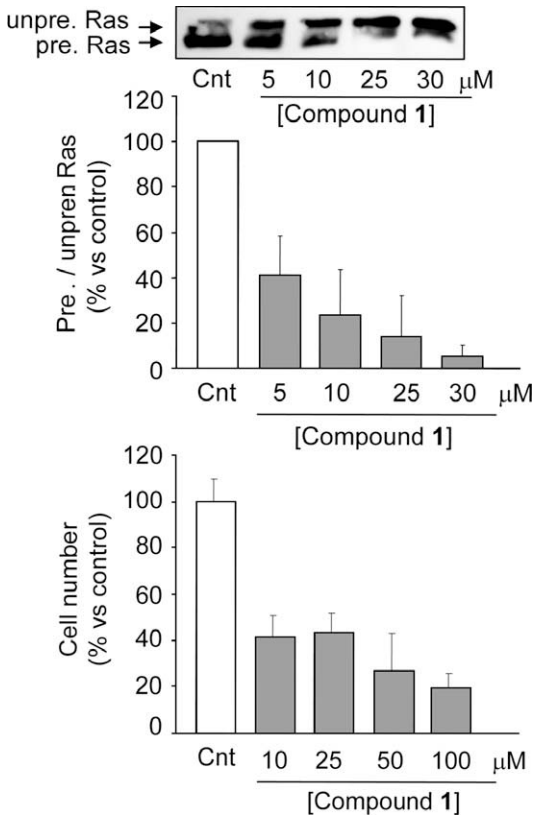


Figure 2. Concentration dependent effect of **1** on Ras prenylation and cell proliferation in human aortic SMCs. Human SMCs were incubated with increasing concentrations of compound **1** for 72 h, total cell lysates were prepared and Ras prenylation evaluated by Western blotting analysis with a specific antibody anti Ras (upstate). The slower migrating band represents the unprenylated form of Ras (unpre. Ras), while the faster migrating band is prenylated Ras (pre. Ras). The proliferation assay was performed under the same experimental conditions and cell number determined after 72 h incubation with indicated concentrations of **1**. CNT: control.

of pyridodioxane and benzodioxane derivatives, we can hypothesize that the present compounds affect only FTase.

In conclusion, we have demonstrated that *CAAX* mimetics, in which C-terminal methionine is esterified or replaced by esterified glycine or leucine and cysteine is replaced by a benzodioxanemethyl residue etherifying the known *AA* bioisostere 2-*o*-tolyl-4-hydroxybenzoic acid exhibit, in whole cell tests, antiproliferative activity at submicromolar or low micromolar level. However, such an activity is associated to a remarkable inhibition of Ras prenylation only in the case of the most potent methionine derivative **1**. Analogously to previous results obtained for pyridodioxane derivatives, critical features are the nature of the linker between dioxane and biphenyl core and the *o*-substitution on the latter, and not the dioxane stereochemistry. Though comparison of the present compounds with the previous ones is limited to **1** and its pyridodioxane analogue, it is significant that, in this case, replacement of pyridodioxane by benzodioxane not only potentiates but also maintains the activity at the highest level in the respective series.

Acknowledgment

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10. 2-(2-Pyrrolidinyl)benzodioxanes (Pallavicini, M.; Bolchi, C.; Binda, M.; Cilia, A.; Clementi, F.; Ferrara, R.; Fumagalli, L.; Gotti, C.; Moretti, M.; Pedretti, A.; Vistoli, G.; Valoti, E. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 854) are much more potent α4β2 nicotinic ligands than 2-(2-pyrrolidinyl)-2,3-dihydro-1,4-dioxino[2,3-*b*]pyridines (unpublished data). WB4101, namely 2-[2-(2,6-dimethoxyphenoxy)ethyl]aminomethyl-1,4-benzodioxane is a more potent α_1 -adrenergic ligand than 2-[2-(2,6-dimethoxyphenoxy)ethyl]aminomethyl-2,3-dihydro-1,4-dioxino[2,3-*b*]pyridine (Valoti, E. Abstracts of papers, XVI Convegno Nazionale Divisione di Chimica Farmaceutica Società Chimica Italiana, Sorrento, Italy, September 2002; Abstract L18).
11. Bolchi, C.; Pallavicini, M.; Fumagalli, L.; Rusconi, C.; Binda, M.; Valoti, E. *Tetrahedron:Asymmetry* **2007**, *18*, 1038.
12. *Compound* **27**: crystallization from cold methanol; mp 83–84 °C; ^{1}H NMR ($CDCl_3$) δ 8.49 (d, J = 2.2 Hz, 1H), 8.19 (dd, J = 8.8, 2.2 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H), 3.98 (s, 3H).
13. *Compound* **28**: LC on silica gel (eluent:cyclohexane/EtOAc 90/10); ^{1}H NMR ($CDCl_3$) δ 8.26 (dd, J = 8.4, 2.2 Hz, 1H), 8.12 (d, J = 2.2 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H), 7.32–7.23 (m, 3H), 7.07 (dd, J = 7.6, 6.6 Hz 1H), 3.65 (s, 3H), 2.09 (s, 3H).
14. *Compound* **29**: crystallization from diisopropyl ether; ^{1}H NMR ($CDCl_3$) δ 8.23 (d, J = 2.2 Hz, 1H), 8.20 (dd, J = 2.3, 2.2 Hz, 1H), 7.97 (d, J = 9.15 Hz, 1H), 7.4 (m, 1H), 7.28 (m, 1H), 7.07 (m, 1H), 6.93 (d, J = 8.05 Hz, 1H), 3.73 (s, 3H), 3.70 (s, 3H).
15. *Compound* **30**: crystallization from diisopropyl ether; ^{1}H NMR ($CDCl_3$) δ 7.88 (d, J = 9.2 Hz, 1H), 7.16–7.25 (m, 3H), 7.05 (d, J = 6.6 Hz, 1H), 6.62–6.66 (m, 1H), 6.43 (d, J = 2.2 Hz, 1H), 4.02 (br s, 2H), 3.57 (s, 3H), 2.07 (s, 3H).
16. *Compound* **31**: crystallization from diisopropyl ether; ^{1}H NMR ($CDCl_3$) δ 7.79 (d, J = 8.4 Hz, 1H), 7.28–7.34 (m, 1H), 7.18 (dd, J = 7.3, 1.8 Hz, 1H), 6.97–7.03 (m, 1H), 6.88 (d, J = 8.1 Hz, 1H), 6.62 (dd, J = 8.4, 2.2 Hz, 1H), 6.54 (d, J = 2.2 Hz, 1H), 3.8–4.2 (br s, 2H), 3.71 (s, 3H), 3.59 (s, 3H).
17. *Compound* **32**: LC on silica gel (eluent:cyclohexane/EtOAc 70/30); ^{1}H NMR ($CDCl_3$) δ 7.95 (d, J = 8.6 Hz, 1H), 7.08–7.23 (m, 3H), 6.95–7.03 (m, 1H), 6.78 (d, J = 8.0 Hz, 1H), 6.6 (s, 1H), 6.3 (s, 1H), 3.59 (s, 3H), 2.05 (s, 3H).
18. *Compound* **33**: LC on silica gel (eluent:cyclohexane/EtOAc 70/30); ^{1}H NMR ($CDCl_3$) δ 7.71 (d, J = 8.4 Hz, 1H), 7.27–7.33 (m, 1H), 7.16 (dd, J = 7.3, 1.8 Hz, 1H), 6.96–7.02 (m, 1H), 6.87 (d, J = 8.05 Hz, 1H), 6.77 (dd, J = 8.4, 2.6 Hz, 1H), 6.71 (d, J = 2.2 Hz, 1H), 5.84 (s, 1H), 3.69 (s, 3H), 3.62 (s, 3H).
19. (*S*)-**34**: LC on silica gel (eluent:cyclohexane/EtOAc 60/40); $[\alpha]_D^{25}$ = −5.80 (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.99 (d, J = 8.8 Hz, 1H), 7.17–7.23 (m, 3H), 7.05 (d, J = 7.0 Hz, 1H), 6.84–6.97 (m, 5H), 6.76 (d, J = 2.9 Hz, 1H), 4.56–4.59 (m, 1H), 4.39 (dd, J = 11.4, 3.1 Hz, 1H), 4.19–4.30 (m, 3H), 3.60 (s, 3H), 2.06 (s, 3H).
20. (*S*)-**35**: LC on silica gel (eluent:cyclohexane/EtOAc 60/40); $[\alpha]_D^{25}$ = −4.0 (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.90 (d, J = 8.8 Hz, 1H), 7.31–7.37 (m, 1H), 7.21 (dd, J = 7.3, 1.8 Hz, 1H), 7.00–7.05 (m, 1H), 6.84–6.94 (m 7H), 4.56–4.59 (m, 1H), 4.39 (dd, J = 11.35, 2.2 Hz, 1H), 4.17–4.25 (m, 3H), 3.71 (s, 3H), 3.63 (s, 3H).
21. (*S*)-**36**: crystallization from isopropyl alcohol; $[\alpha]_D^{25}$ = −32.0 (*c* 1, methanol); ^{1}H NMR (DMSO-d_6) δ 12.19 (br s, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.02–7.22 (m, 5H), 6.78–6.99 (m, 4H), 6.71 (d, J = 2.6 Hz, 1H), 4.52–4.59 (m, 1H), 4.25–4.42 (m, 3H), 4.12 (dd, J = 11.7, 7.3 Hz, 1H), 2.00 (s, 3H).
22. (*S*)-**37**: crystallization from diisopropyl ether/isopropyl alcohol 1/1; $[\alpha]_D^{25}$ = −28.5 (*c* 1, methanol); ^{1}H NMR (DMSO-d_6) δ 12.07 (s, 1H), 7.76 (d, J = 8.8 Hz, 1H), 7.25–7.31 (m, 1H), 7.16 (dd, J = 7.7, 1.8 Hz, 1H), 6.78–7.02 (m, 8H), 4.54–4.57 (m, 1H), 4.41 (dd, J = 11.35, 2.2 Hz, 1H), 4.27–4.36 (m, 2H), 4.12 (dd, J = 11.7, 7.3 Hz, 1H), 3.62 (s, 3H).
23. *2(S)-[2-(o-tolyl)-4-(2(S)-1,4-benzodiossan-2-ylmethoxy)benzamido]-4-methylthiobutyric acid methyl ester* (**1**): LC on silica gel (eluent:cyclohexane/ethyl acetate 60/40); $[\alpha]_D^{25}$ = +8.9 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **2**.
24. *[2-(o-tolyl)-4-(2(S)-1,4-benzodioxan-2-ylmethoxy)benzamido]acetic acid methyl ester* (**5**): LC on silica gel (eluent:cyclohexane/ethyl acetate 60/40); $[\alpha]_D^{25}$ = −6.6 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **6**.
25. *2(S)-[2-(o-tolyl)-4-(2(S)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylvaleric acid methyl ester* (**9**): LC on silica gel (eluent:cyclohexane/ethyl acetate 70/30); $[\alpha]_D^{25}$ = +8.5 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **10**.
26. *2(S)-[2-(o-methoxyphenyl)-4-(2(S)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylthio-butyric acid methyl ester* (**3**): LC on silica gel (eluent:cyclohexane/ethyl acetate 1/1); $[\alpha]_D^{25}$ = +18.4 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **4**.
27. *[2-(o-methoxyphenyl)-4-(2(S)-1,4-benzodioxan-2-ylmethoxy)benzamido]acetic acid methyl ester* (**7**): LC on silica gel (eluent:cyclohexane/ethyl acetate 40/60); $[\alpha]_D^{25}$ = −4.30 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **8**.
28. *2(S)-[2-(o-methoxyphenyl)-4-(2(S)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylvaleric acid methyl ester* (**11**): LC on silica gel (eluent:cyclohexane/ethyl acetate 60/40); $[\alpha]_D^{25}$ = +10.7 (*c* 1, $CHCl_3$); ^{1}H NMR consistent with that of **12**.

29. (*R*)-**34**: $[\alpha]_D^{25}$ = +5.0 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**34**.
30. (*R*)-**35**: $[\alpha]_D^{25}$ = +4.4 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**35**.
31. (*R*)-**36**: $[\alpha]_D^{25}$ = +30.7 (*c* 1, methanol); ^1H NMR identical to that of (*S*)-**36**.
32. (*R*)-**37**: $[\alpha]_D^{25}$ = +29.1 (*c* 1, methanol); ^1H NMR identical to that of (*S*)-**37**.
33. *2(S)-[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylthiobutyric acid methyl ester* (**2**): $[\alpha]_D^{25}$ = +19.0 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.93 (dd, *J* = 18.75, 8.8 Hz, 1H), 7.09–7.27 (m, 5H), 6.92 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.75–6.84 (m, 4H), 6.63 (d, *J* = 2.6 Hz, 1H), 5.74 (d, *J* = 7.3 Hz, 1H), 4.47–4.55 (m, 2H), 4.32 (dd, *J* = 11.4, 2.35 Hz, 1H), 4.12–4.24 (m, 3H), 3.57 (s, 3H), 1.93–2.11 (m, 7H), 1.71–1.82 (m, 1H), 1.42–1.54 (m, 2H).
34. *[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]acetic acid methyl ester* (**6**): $[\alpha]_D^{25}$ = +4.3 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.01 (d, *J* = 8.8 Hz, 1H), 7.20–7.32 (m, 4H), 6.99 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.83–6.92 (m, 4H), 6.71 (d, *J* = 2.6 Hz, 1H), 5.81 (br s, 1H), 4.56–4.59 (m, 1H), 4.39 (dd, *J* = 11. 35, 2.2 Hz, 1H), 4.19–4.28 (m, 3H), 3.99 (d, *J* = 5.1 Hz, 2H), 3.65 (s, 3H), 2.12 (s, 3H).
35. *2(S)-[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylvaleric acid methyl ester* (**10**): $[\alpha]_D^{25}$ = +17.9 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.05 (dd, *J* = 22.3, 8.8 Hz, 1H), 7.16–7.33 (m, 3H), 6.99 (dd, *J* = 88.8, 2.6 Hz, 1H), 6.83–6.92 (m, 5H), 6.70 (t, *J* = 2.2 Hz, 1H), 5.59 (br s, 1H), 4.37–4.60 (m, 3H), 4.08–4.31 (m, 3H), 3.63 (s, 3H), 2.05 (s, 3H), 0.92–1.26 (m, 3H), 0.74–0.80 (m, 6H).
36. *2(S)-[2-(o-methoxyphenyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylthio-butyric acid methyl ester* (**4**): $[\alpha]_D^{25}$ = +25.3 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.78 (d, *J* = 8.4 Hz, 1H), 7.36–7.41 (m, 1H), 7.21 (d, *J* = 6.2 Hz, 1H), 6.83–7.03 (m, 7H), 6.76 (d, *J* = 2.6 Hz, 1H), 6.25 (br s, 1H), 4.55–4.68 (m, 2H), 4.39 (dd, *J* = 11.7, 2.6 Hz, 1H), 4.18–4.31 (m, 3H), 3.78 (s, 3H), 3.66 (s, 3H).
37. *[2-(o-methoxyphenyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]acetic acid methyl ester* (**8**): $[\alpha]_D^{25}$ = +4.0 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.80 (d. *J* = 8.8 Hz, 1H), 7.26–7.39 (m, 1H), 7.23 (dd, *J* = 7.3, 1.8 Hz, 1H), 6.80–7.06 (m, 8H), 6.09 (br s, 1H), 4.53–4.58 (m, 1H), 4.26–4.31 (m, 1H), 4.11–4.24 (m, 3H), 3.94 (d, *J* = 5.1 Hz, 2H), 3.76 (s, 3H), 3.66 (s, 3H).
38. *2(S)-[2-(o-methoxyphenyl)-4-(2(R)-1,4-benzodioxan-2-ylmethoxy)benzamido]-4-methylvaleric acid methyl ester* (**12**): $[\alpha]_D^{25}$ = +18.5 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.74 (d, *J* = 8.8 Hz, 1H), 7.27–7.33 (m, 1H), 7.13 (d, *J* = 2.05 Hz, 1H), 6.75–6.97 (m, 7H), 6.68 (d, *J* = 1.2 Hz, 1H), 5.94 (br s, 1H), 4.42–4.51 (m, 2H), 4.31 (dd, *J* = 11.4, 2.35 Hz, 1H), 4.08–4.23 (m, 3H), 3.69 (s, 3H), 3.56 (s, 3H), 0.96–1.37 (m, 3H), 0.67–0.73 (dd, *J* = 12.0, 6.4 Hz, 6H).
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40. Bolchi, C.; Catalano, P.; Fumagalli, L.; Gobbi, M.; Pallavicini, M.; Pedretti, A.; Villa, L.; Vistoli, G.; Valoti, E. *Bioorg. Med. Chem.* **2004**, *12*, 4937.
41. (*S*)-**39**: LC on silica gel (eluent:cyclohexane/EtOAc 70/30); $[\alpha]_D^{25}$ = −35.6 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.01 (d, *J* = 8.05 Hz, 1H), 7.83 (dd, *J* = 8.05, 1.8 Hz, 1H), 7.62 (d, *J* = 1.5 Hz, 1H), 7.18–7.31 (m, 3H), 7.06 (d, *J* = 7.3 Hz, 1H), 6.81–6.89 (m, 5H), 6.68 (t, J = 5.9 Hz, 1H), 4.37–4.42 (m, 1H), 4.32 (dd, *J* = 11.35, 2.2 Hz, 1H), 4.03 (dd, *J* = 11.35, 7.3 Hz, 1H), 3.82–3.88 (m, 1H), 3.65–3.75 (m, 1H), 3.63 (s, 3H), 2.06 (s, 3H).
42. (*S*)-**41**: crystallization from diisopropyl ether; $[\alpha]_D^{25}$ = −34.5 (*c* 1, $CHCl_3$); ^1H NMR (DMSO-d_6) δ 12.83 (br s, 1H), 8.93 (t, *J* = 5.9 Hz, 1H), 7.88–7.96 (m, 2H), 7.71 (d, *J* = 1.1 Hz, 1H), 7.17–7.24 (m, 3H), 7.06 (d, *J* = 6.95 Hz, 1H), 6.77–6.86 (m, 4H), 4.28–4.34 (m, 2H), 3.96–4.00 (m, 1H), 3.51–3.57 (m, 2H), 2.01 (s, 3H).
43. *2(S)-[2-(o-tolyl)-4-(2(S)-1,4-benzodioxan-2-ylmethylaminocarbonyl)benzamido]-4-methylthio-butyric acid methyl ester* (**13**): LC on silica gel (eluent:cyclohexane/ethyl acetate 1/1); $[\alpha]_D^{25}$ = −4.8 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **14**.
44. *[2-(o-tolyl)-4-(2(S)-1,4-benzodioxan-2-ylmethylamino carbonyl)benzamido]acetic acid methyl ester* (**17**): LC on silica gel (eluent:cyclohexane/ethyl acetate 40/60); $[\alpha]_D^{25}$ = −40.1 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **18**.
45. *2(S)-[2-(o-tolyl)-4-(2(S)-1,4-benzodioxan-2-ylmethyl aminocarbonyl)benzamido]-4-methylvaleric acid methyl ester* (**21**): LC on silica gel (eluent:cyclohexane/ethyl acetate 60/40); $[\alpha]_D^{25}$ = −11.0 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **22**.
46. (*S*)-**40**: LC on silica gel (eluent: cyclohexane/EtOAc 70/30); $[\alpha]_D^{25}$ = −32.3 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.90 (d, *J* = 7.7 Hz, 1H), 7.78 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.71 (d, *J* = 1.8 Hz, 1H), 7.32–7.38 (m, 1H), 7.24–7.27 (m, 1H), 7.02–7.07 (m, 1H), 6.82–6.91 (m, 5H), 6.69 (br s, 1H), 4.30–4.40 (m, 2H), 4.00 (dd, *J* = 11.35, 6.95 Hz, 1H), 3.82–3.91 (m, 1H), 3.70 (s, 3H), 3.67 (s, 3H).
47. (*S*)-**42**: crystallization from diisopropyl ether; m.p. 72 °C; $[\alpha]_D^{25}$ = −31.5 (*c* 1, $CHCl_3$); ^1H NMR (DMSO-d_6) δ 12.64 (br s, 1H), 8.93 (pseudo t, 1H), 7.78–7.90 (m, 3H), 7.25–7.35 (m, 2H), 6.98–7.04 (pseudo t, 1H), 6.78–6.87 (m, 4H), 4.29–4.35 (m, 2H), 3.95–4.01 (m, 1H), 3.64 (s, 3H), 3.52–3.60 (m, 2H).
48. *2(S)-[2-(o-methoxyphenyll)-4-(2(S)-1,4-benzodioxan-2-ylmethylaminocarbonyl)-benzamido]-4-methylthio-butyric acid methyl ester* (**15**): LC on silica gel (eluent: cyclohexane/ethyl acetate 1/1); $[\alpha]_D^{25}$ = +6.6 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **16**.
49. *[2-(o-methoxyphenyll)-4-(2(S)-1,4-benzodioxan-2-ylmethylaminocarbonyl)benz-amido]acetic acid methyl ester* (**19**): LC on silica gel (eluent:cyclohexane/ethyl acetate 40/60); $[\alpha]_D^{25}$ = −33.0 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **20**.
50. *2(S)-[2-(o-methoxyphenyll)-4-(2(S)-1,4-benzodioxan-2-ylmethylaminocarbonyl)-benzamido]-4-methyl valeric acid methyl ester* (**23**): LC on silica gel (eluent:cyclohexane/ethyl acetate 60/40); $[\alpha]_D^{25}$ = −4.5 (*c* 1, $CHCl_3$); ^1H NMR consistent with that of **24**.
51. (*R*)-**39**: $[\alpha]_D^{25}$ = +36.7 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**39**.
52. (*R*)-**40**: $[\alpha]_D^{25}$ = +36.9 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**40**.
53. (*R*)-**41**: $[\alpha]_D^{25}$ = +33.4 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**41**.
54. (*R*)-**42**: $[\alpha]_D^{25}$ = +32.3 (*c* 1, $CHCl_3$); ^1H NMR identical to that of (*S*)-**42**.
55. *2(S)-[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethylaminocarbonyl)benzamido]-4-methylthio-butyric acid methyl ester* (**14**): $[\alpha]_D^{25}$ = +46.8(*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.95–8.03 (m, 1H), 7.82 (d, *J* = 8.05 Hz, 1H), 7.63 (s, 1H), 7.16–7.36 (m, 3H), 6.82–6.90 (m, 4H), 6.66–6.70 (m, 1H), 5.96–6.00 (m, 1H), 4.58–4.65 (m, 1H), 4.31–4.42 (m, 2H), 4.01 (dd, *J* = 11.35, 7.3 Hz, 1H), 3.84–3.93 (m, 1H), 3.69–3.75 (m, 1H), 3.66 (s, 3H), 1.99–2.17 (m, 8H), 1.82–1.89 (m, 1H), 1.59–1.65 (m, 1H).
56. *[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethylaminocarbonyl)benzamido]acetic acid methyl ester* (**18**): $[\alpha]_D^{25}$ = +31.2 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.02 (d, *J* = 8.2 Hz, 1H), 7.82 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.64 (d, *J* = 1.8 Hz, 1H), 7.19–7.33 (m, 4H), 6.82–6.90 (m, 4H), 6.67 (br s, 1H), 5.95 (br s, 1H), 4.38–4.41 (m, 1H), 4.33 (dd, *J* = 11.4, 2.3 Hz, 1H), 4.01 (dd, *J* = 11.4, 7.3 Hz, 1H), 3.91 (d, *J* = 5.3 Hz, 2H), 3.85–3.88 (m, 1H), 3.68–3.75 (m, 1H), 3.66 (s, 3H), 2.11 (s, 3H).
57. *2(S)-[2-(o-tolyl)-4-(2(R)-1,4-benzodioxan-2-ylmethyl aminocarbonyl)benzamido]-4-methylvaleric acid methyl ester* (**22**): $[\alpha]_D^{25}$ = +40.9 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.99–8.05 (d, *J* = 8.6 Hz, 1H), 7.80 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.63 (s, 1 H), 7.15–7.33 (m, 4H), 6.81–6.89 (m, 4H), 6.73 (t, *J* = 5.9 Hz, 1H), 5.76 (d, *J* = 7.7 Hz, 1H), 4.31–4.42 (m, 3H), 3.97–4.03 (m, 1H), 3.83–3.92 (m, 1H), 3.66–3.75 (m, 1H), 3.64 (s, 3H), 2.05 (s, 3H), 1.25–1.33 (m, 1H), 0.97–1.06 (m, 2H), 0.76 (pseudo t, 6H).
58. *2(S)-[2-(o-methoxyphenyll)-4-(2(R)-1,4-benzodioxan-2-ylmethylaminocarbonyl)-benzamido]-4-methylthio-butyric acid methyl ester* (**16**): $[\alpha]_D^{25}$ = +54.7 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.79 (d, *J* = 0.7 Hz, 1H), 7.65 (d, *J* = 1.1 Hz, 1H), 7.37–7.43 (m, 1H), 7.22 (d, *J* = 6.6 Hz, 1H), 7.01–7.07 (m, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.81–6.89 (m, 3H), 6.70 (m, 1H), 6.50 (br s, 1H), 4.63–4.68 (m, 1H), 4.30–4.39 (m, 2H), 3.96–4.10 (m, 1H), 3.82–3.90 (m, 1H), 3.77 (s, 3H), 3.69–3.75 (m, 1H), 3.66 (s, 3H), 1.87–2.16 (m, 5H), 1.65–1.67 (m, 2H).
59. *[2-(o-methoxyphenyll)-4-(2(R)-1,4-benzodioxan-2-ylmethylaminocarbonyl)benz-amido]acetic acid methyl ester* (**20**): $[\alpha]_D^{25}$ = −33.0 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.69–7.76 (m, 2H), 7.61 (d, *J* = 1.8 Hz, 1H), 7.28–7.34 (m, 1H), 7.17 (dd, *J* = 6.95, 1.8 Hz, 1H), 6.94–6.99 (m, 1H), 6.82–6.88 (m, 1H), 6.74–6.81 (m, 4H), 6.63 (t, *J* = 5.9 Hz, 1H), 6.21 (t, *J* = 4.8 Hz, 1H), 4.23–4.34 (m, 2H), 3.75–4.05 (m, 4H), 3.68 (s, 3H), 3.60 (s, 3H).
60. *2(S)-[2-(o-methoxyphenyll)-4-(2(S)-1,4-benzodioxan-2-ylmethylaminocarbonyl)-benzamido]-4-methyl valeric acid methyl ester* (**24**): $[\alpha]_D^{25}$ = +51.7 (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.73 (d, *J* = 8.05 Hz, 1H), 7.78 (dd, *J* = 8.05, 1.5 Hz, 1H), 7.65 (d, *J* = 1.5 Hz, 1H), 7.36–7.42 (m, 1H), 7.20–7.23 (m, 1H), 7.03 (t, *J* = 7.3 Hz, 1H), 6.94 (d, *J* = 8.4 Hz, 1H), 6.91–6.96 (m, 4H), 6.65 (br s, 1H), 6.20 (br s, 1H), 4.51–4.57 (m, 1H), 4.30–4.39 (m, 2H), 3.97–4.03 (m, 1H), 3.83–3.91 (m, 1H), 3.75 (s, 3H), 3.65–3.73 (m, 1H), 3.64 (s, 3H), 1.33–1.37 (m, 1H), 1.09–1.16 (m, 2H), 0.74–0.80 (2 d, 6H).
61. *Cell proliferation assay*. Human aortic SMCs were seeded at a density of 8×10^4 cells/Petri dish (35 mm), and incubated with DMEM supplemented with 10% FCS. Twenty-four hours later, the medium was changed to one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 72 h. At this time (time 0), the medium was replaced with one containing 10% FCS in the presence or absence of known concentrations of the tested compounds, and the incubation was continued for a further 72 h at 37 °C. Cell proliferation was evaluated by cell counting after trypsinization of the monolayers with use of a Coulter Counter model ZM (Corsini, A.; Mazzotti, M.; Raiteri, M.; Soma, M. R.; Gabbiani, G.; Fumagalli, R.; Paoletti, R. *Atherosclerosis* **1993**, *101*, 117). All the compounds were dissolved in DMSO prior to dilution, being the final concentration of DMSO at a maximum of 0.5%. The final concentration of DMSO was normalized across all the different conditions. The concentration of compounds required to inhibit 50% of cell proliferation (IC_{50}) was calculated by linear regression analysis of the logarithm of the concentration versus logit by using the SigmaPlot 8.0 software.
62. *Ras prenylation assay*. HSMCs were seeded at a density of 4×10^5 cells/Petri dish (60 mm) and incubated under the same experimental conditions described for the cell proliferation assay. At the end of the 72 h of incubation with the tested compounds, cells were washed twice with cold phosphate buffer saline (PBS) and lysed in 200 μl buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 1 mM $NaVO_4$, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin and 1 μg/ml Pepstatin) for 30 min on ice. Cell lysates were cleared by centrifugation at 15,000 g for 10 min, and protein concentrations were determined using the BCA protein assay (Pierce). Lysates were separated by SDS-PAGE under reducing conditions, transferred to Immobilon PVDF (Millipore) and subsequently immunoblotted with anti Ras antibody (Upstate), prior to visualization by enhanced chemiluminescence (ECL, Amersham Corp.) (Ferri, N.; Colombo, G.; Ferrandi, C.; Raines, E.W.; Levkau, B.; Corsini, A. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 1043).
63. Human aortic SMCs express all three isoforms of Ras (H-Ras, K-Ras, and N-Ras). The antibody utilized for the detection of Ras by western blotting analysis recognizes all three isoforms and therefore the isoform specific effect of **1** on Ras prenylation could not be studied. Nonetheless, the almost complete inhibition of Ras prenylation by **1** indicates that all three isoforms were affected by this compound.
64. Compounds **5**–**12** were tested for their ability to inhibit Ras prenylation at a fixed concentration that elicited the highest non-toxic antiproliferative activity (10 μM for **9** and **12**; 25 μM for **6**, **10** and **11**; 50 μM for **5**; 75 μM for **7** and **8**). At this concentration, none of the compounds was found to significantly affect Ras prenylation excepting **5** (35% inhibition). Since the incubation with higher concentrations would elicit a toxic effect, we could not determine Ras prenylation inhibition and respective IC_{50} value for these compounds.