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Design, Synthesis, and Biological Evaluation of Levoglucosenone-Derived Ras Activation Inhibitors

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Ras is an essential component of signal transduction pathways that regulate cell growth, proliferation, differentiation, and apoptosis.[1] Ras functions as a molecular switch by cycling between an active GTP-bound form and an inactive GDP-bound form. The conversion from the GTP-bound to the GDP-bound form is accelerated by binding to GTPase-activating proteins (GAPs), while the GDP-to-GTP exchange is catalyzed by guanine nucleotide exchange factors (GEFs).[2] Impairment of the hydrolytic reaction is the common biochemical defect associated with oncogenic Ras mutations.[3] Ras mutants are expressed in more than 30% of human tumors. These mutants are constitutively active and are insensitive to GAP catalysis, and thus they remain in the GTP-bound state for an abnormally prolonged period. The search for small molecules with druglike characteristics that can efficiently revert the constitutive activation of Ras remains a very challenging and unsolved task in the quest to develop highly selective anticancer agents. Rational design and combinatorial approaches have allowed the identification of low-molecular-weight active compounds that target the Ras activation process at various levels: inhibitors of farnesyl-, geranylgeranyl-, and palmitoyltransferases,[4] GTP analogues activated to undergo spontaneous phosphate hydrolysis which should compensate for the very low GTPase activity of oncogenic Ras mutants,[5] and inhibitors of the interaction between Ras and the downstream effector Raf.[6] Inspired by the pioneering work of researchers at Schering-Plough,[7] we proposed an alternative approach to anticancer drug development based on small organic molecules that inhibit the GEF-promoted nucleotide exchange on Ras.[8] Molecules composed of two aromatic moieties connected by either a linear linker or an arabinose- or glucose-derived scaffold were synthesized, and their biological activity was tested. NMR saturation transfer difference (STD) and trNOESY experiments in solution showed that these compounds bind Ras, with the aromatic groups playing a prominent role in the interaction with the protein. The presence of two aromatic moieties is important not only for Ras binding but also for inhibiting the GTP/GDP exchange on Ras in vitro. Surface plasmon resonance (SPR) experiments showed that arabinose- and glucose-derived molecules interfere with the Ras–GEF (Cdc25) interaction, and this would very likely decrease the rate of GEF-catalyzed nucleotide exchange.[9] All Ras ligands developed so far contain a phenylhydroxylamine moiety that is involved in important interactions with Ras residues, but that is unsuitable for drug development because of its low chemical stability and its production of toxic metabolites in vivo.

Our general aim was to create new Ras ligands by functionalizing scaffolds derived from natural products with hydroxy-containing moieties. In particular, we present herein the synthesis and biological evaluation of compounds **1**–**9**, which have a common tricyclic scaffold derived from the 1,3-dipolar cycloaddition of aromatic nitrones to levoglucosenone (Figure 1). Levoglucosenone is a versatile carbohydrate-derived structure with a reactive moiety and a rigid, nonplanar cyclic architecture.[10] The α,β-unsaturated carbonyl group can undergo several different reactions, particularly cycloadditions, such as 1,3-dipolar cycloadditions with nitrones to afford tricyclic structures (Figure 1a) with a high degree of regio- and stereoselectivity.[11–13] The tricyclic scaffold consists of the levoglucosane system fused with an isoxazolidine ring. The presence of five heteroatoms and the possibility of projecting potential pharmacophore groups into defined regions of space make the tri-heterocycle an attractive scaffold for the creation of pharmacologically active compounds. Molecular modeling and virtual ligand docking were applied in the design of new Ras inhibitors based on the tricyclic scaffold as outlined in Figure 1a. We started with the design of a focused virtual library by varying substituents R^1, R^2, and R^3 with insight gathered from earlier studies[8] to avoid generating a random virtual library with an accompanying combinatorial explosion. R^1 and R^2 were chosen to be phenyl derivatives; however, either R^1 or R^2 is always a phenyl group, whereas the other is a functionalized *m*- or *p*-biphenyl derivative. The functional groups on the *m*- or *p*-biphenyl moieties were designed to replace the toxic *p*-hydroxylamine group of the previously published arabinose- and glucose-derived Ras inhibitors[8–9] without sacrificing too much of the beneficial interactions with Ras. Several substituents were screened: halogen, dihalogen, methoxy, trifluoromethoxy, hydroxymethyl, dihydroxyethyl, dioxolane, nitro groups, or other related disubstitutions. R^3 can be either =O (as in compounds **1**–**5**), –OH, –OEt, or $-OCH_2Ph$ (as in compounds

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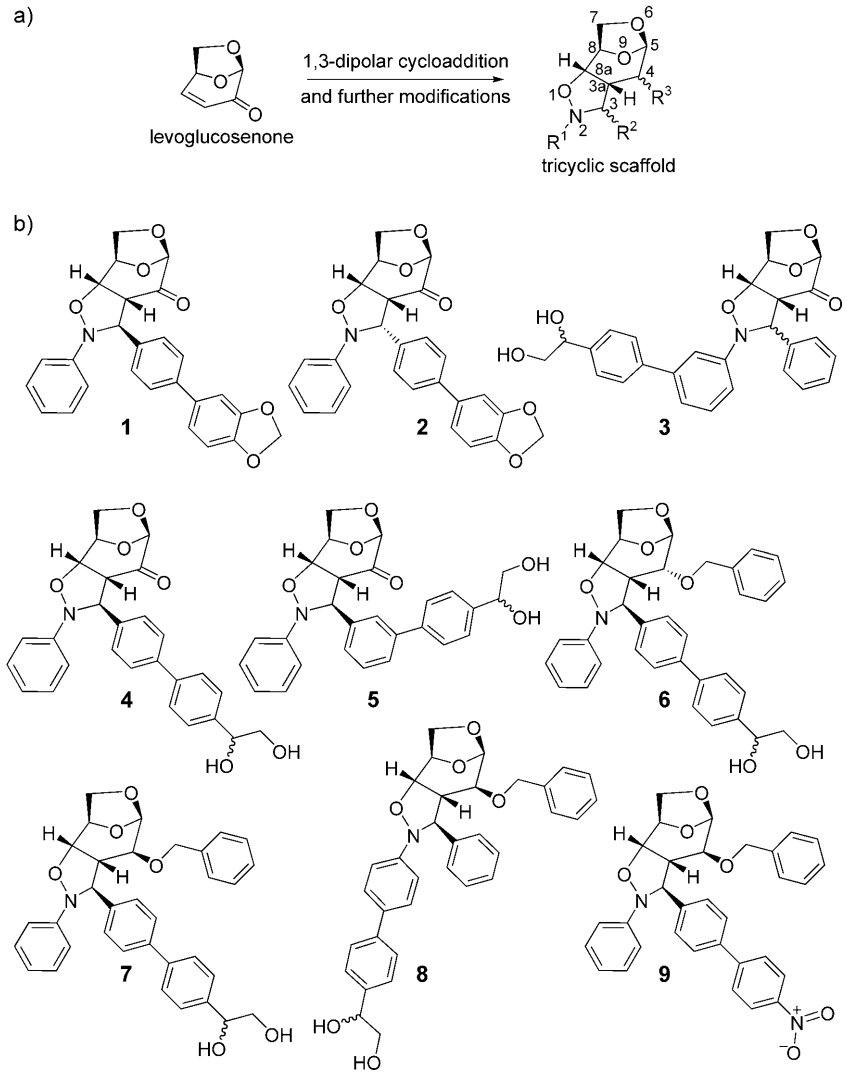
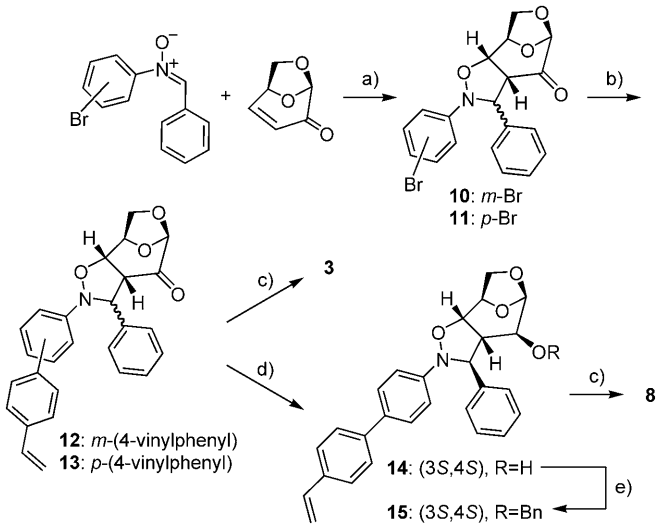


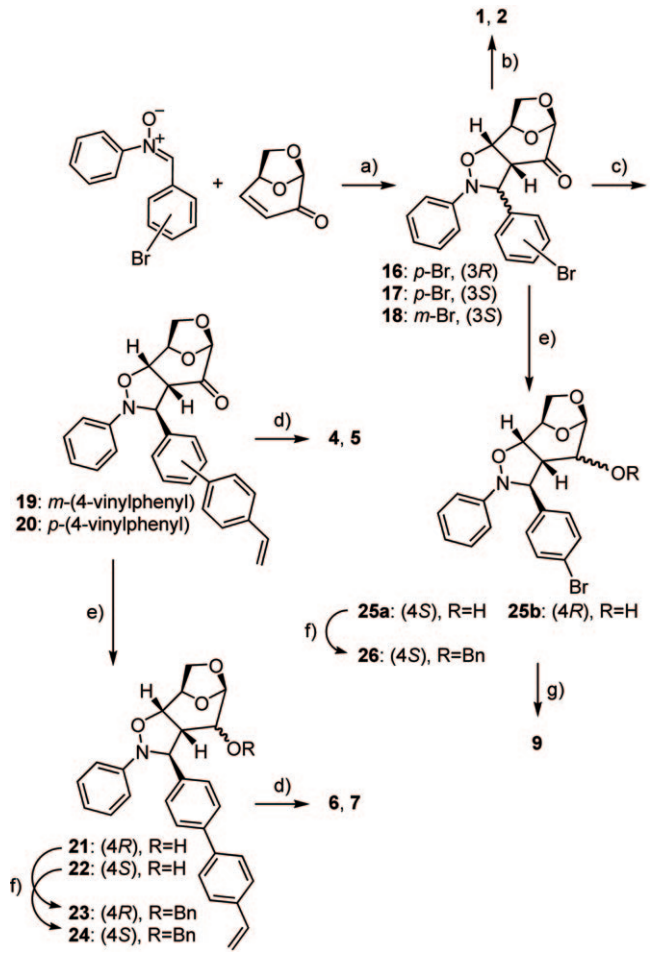
Figure 1. a) Generation of a tricyclic scaffold by 1,3-dipolar addition to levoglucosenone. b) Structures of compounds **1**–**9**.

6–**9** to favor lipophilicity and hence cell penetration). All possible stereoisomers were generated. The focused library was screened in silico as described in the Supporting Information. On the basis of calculated binding affinity, calculated binding mode, and synthetic feasibility, compounds **1**–**9** were finally selected. Several of the aforementioned moieties (such as halogen, dihalogen, methoxy, trifluoromethoxy, and monohydroxy) were not computationally favored, and we therefore discarded them. The dioxolane moiety had a medium virtual binding affinity as listed in table 2s in the Supporting Information (this group is present in compounds **1** and **2**). The moieties favored computationally were *m*,*p*-disubstituted biphenyls with two –OH groups (catechol) or –OH and $-NH_2$, but for the moment we excluded these groups because of a greater complexity in the synthesis. Biphenyl groups substituted *para* at the terminal phenyl ring with the dihydroxyethyl moiety (compounds **3**–**8**), were also favored, with a preferred *S* configuration at C3. Compounds **1** and **2** are diastereomers bearing a *p*-substituted biphenyl moiety at C3 with *S* and *R* absolute configurations, respectively. The functional group present in **1** and **2** is a catechol-formaldehyde acetal. In compounds **3**–**8**, a vicinal aliphatic diol has been inserted at the *para* position of the terminal phenyl group. The regiochemistry of the biphenyl substitution (*meta* or *para*) and the point of attachment on the scaffold (C3 or N2) vary in these compounds. In compound **9** there is a nitro group at the *para* position of the terminal aromatic ring. When R^3 is a benzyl ether group (compounds **6**–**9**) instead of a carbonyl group (compounds **1**–**5**), a lipophilic aromatic group is added that should favor cell penetration, and an additional point of interaction with the protein can be observed in docking experiments. For compounds **6**–**9**, two binding modes are adopted in silico based on rotation along the R^2 axis (which is directed toward the Mg^{2+} ion bound to Ras) with only little difference in binding affinity. Interestingly, with the exception of compound **8**, the top ligand poses bind to the protein with the benzyl ether group at R^3.

Compounds **1**–**9** were obtained by elaborating the aromatic moieties of common precursors derived by 1,3-dipolar addition of aromatic nitrones on levoglucosenone, as depicted in Scheme 1 and Scheme 2. The nitrones were prepared by the reaction of aromatic aldehydes with arylhydroxylamines, which in turn were obtained by reduction of the corresponding nitroaryl group with zinc powder in ethanol in the presence of ammonium chloride or acetic acid.[13] The aromatic nitrone and levoglucosenone reacted in the 1,3-dipolar cycloaddition to give the tricyclic scaffold hexahydro-5,8-epoxyoxepino[4,5-*d*]isoxazol-4(5*H*)-one, as shown in the schemes. The cycloaddition was completely regioselective, with the oxygen attack at the β-carbon atom of the unsaturated ketone due to the polarization of the double bond. Stereoselectivity at the two carbon atoms (C3a and C8a) common to the two fused rings was also complete. The stereoselectivity at C3 depends on the structure of the nitrone substrate and reaction conditions (solvent, temperature, Lewis acid), and it is determined by the relative proportion of exo- and endo-cycloaddition products, respectively giving rise to 3*R* and 3*S* configurations.[14] In the case of the nitrone derived from *p*-bromobenzaldehyde (Scheme 2), a mix-



Scheme 1. *Reagents and conditions*: a) $ZnCl_2{\cdot}Et_2O$, CH_2Cl_2, RT, 8 h; b) 4-vinylphenylboronic acid, K_2CO_3, [Pd(dppf)Cl_2] (cat), DMF/H_2O, 75 °C, 5 h; c) OsO_4, NMO·H_2O, *t*BuOH/H_2O/CH_3CN, RT; d) 1 M DIBAL-H in CH_2Cl_2, 0 °C, 1 h; e) $PhCH_2Br$, NaH, DMF, overnight.



Scheme 2. *Reagents and conditions*: a) $ZnCl_2{\cdot}Et_2O$, CH_2Cl_2, RT, 8 h; b) (1,3-benzodioxol-5-yl)boronic acid, K_2CO_3, [Pd(dppf)Cl_2] (cat), DMF/H_2O, 75 °C, 6 h; c) 4-vinylphenylboronic acid, K_2CO_3, [Pd(dppf)Cl_2] (cat), DMF/H_2O, 75 °C, 5 h; d) OsO_4, NMO·H_2O, *t*BuOH/H_2O/CH_3CN, RT; e) 1 M DIBAL-H in CH_2Cl_2, 0 °C, 1 h; f) $PhCH_2Br$, NaH, DMF, overnight.

ture of 3*R* and 3*S* diastereomers was obtained at a 1:1 ratio when the cycloaddition was performed in dichloromethane at room temperature in the presence of zinc chloride as a Lewis acid promoter. The two bromo derivative diastereomers were separated by column chromatography. The bromophenyl group of each diastereomer was then transformed into a biphenyl group through palladium-catalyzed Suzuki coupling with the appropriately functionalized boronic acid. Reaction with (1,3-benzodioxol-5-yl)boronic acid in the presence of [Pd(dppf)Cl_2] and a base directly provided diastereomers **1** and **2**. The commercially available *p*-vinylphenylboronic acid was used in Suzuki coupling for access to derivatives **4** and **5** (Scheme 2). The vinyl group was then converted into the final diol by treatment with osmium tetroxide in the presence of *N*-methylmorpholine-*N*-oxide (NMO). When starting from the 3*S* diastereomer **17**, the absolute configuration at C3 was preserved during both Suzuki coupling and osmium-mediated oxidation. Compounds **1** and **4** were obtained from **17** as pure 3*S* diastereomers; compound **5** also retained the absolute 3*S* configuration of the starting *m*-bromo derivative **18** (Scheme 2). Starting from the 3*R* diastereomer **16**, epimerization took place during the long thermal treatment required for Suzuki coupling to give a 1:1 *R*/*S* mixture of biphenyl derivatives. The epimerization in favor of the more thermodynamically stable *S* isomer has been investigated and proven in other cases by our group as well (unpublished results). Compounds **3** and **8** were obtained by starting from *N*-*m*-bromophenyl- and *N*-*p*-bromophenylnitrones, respectively (Scheme 1). The nitrones were obtained by condensation of benzaldehyde with 3-bromo- or 4-bromophenylhydroxylamine, which in turn were prepared by reduction of the corresponding nitro derivatives by treating with zinc powder in ethanol in the presence of ammonium chloride or acetic acid. Analogously, after the 1,3-dipolar cycloaddition with levoglucosenone, the *N*-bromoarylisoxazolidine adducts **10** and **11** underwent Suzuki coupling with *p*-vinylphenylboronic acid to give compounds **12** and **13**. OsO_4-mediated dihydroxylation of **12** provided compound **3** (Scheme 1). To obtain the 4-benzyloxy derivatives **6**, **7**, and **8**, we decided to carry out the ketone reduction of the vinylbiphenyl derivative by using diisobutylaluminum hydride (DIBAL-H), and obtained a separable mixture of the corresponding (4*R*) and (4*S*) alcohols. Once generated, alcohols **21** and **22** were converted into the corresponding benzyl ethers **23** and **24**, and subsequently treated with catalytic OsO_4 and NMO to obtain compounds **6** and **7** (Scheme 2). Reduction of ketone **13**, successive benzylation, and final dihydroxylation provided compound **8** (Scheme 1). In the case of the *p*-nitroaryl derivative **9**, a different synthetic way was followed because the Suzuki reaction of compounds **16** and **17** with *p*-nitrophenylboronic acid gave a complex mixture of products (Scheme 2). In this case it was more convenient to first perform carbonyl reduction to **25**, etherification to **26**, and finally Suzuki coupling to obtain compound **9**.

Compounds **1**–**9** were first tested in vitro to investigate their ability to inhibit the C-Cdc25Mm-stimulated nucleotide exchange on purified human Ras protein ($p21^{hRas}$) (Figure 2). For this purpose, a modified version of the method reported by

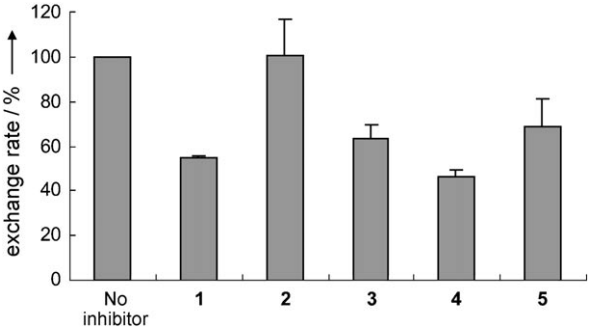


Figure 2. Inhibition of C-Cdc25Mm-stimulated nucleotide exchange on $p21^{hRas}$: Values are expressed as a percentage of the control exchange rate. All compounds were tested at a concentration of 50 μM.

Lenzen et al. was used.[15] C-Cdc25Mm-stimulated guanine nucleotide exchange was monitored in the presence of excess fluorescent 2′(3′)-*O*-(*N*-methylanthraniloyl)-GTP (mant-GTP); $p21^{hRas}$ was incubated with mant-GTP in the absence or presence of compounds **1**–**9** at a final concentration of 50 μM. The exchange reaction was started by the addition of C-Cdc25Mm. Benzyloxy derivatives **6**–**9**, with a further aromatic moiety at C4, were sparingly soluble in the buffer used for the nucleotide exchange experiments. The resulting solutions were turbid after compound addition, thus hampering a reproducible determination of fluorescence. Compounds **1** and **4** were the most potent inhibitors, as the Cdc25-stimulated nucleotide exchange on $p21^{hRas}$ in the presence of these molecules was significantly lowered. Compounds **5** and **3** were less active, while compound **2** was completely inactive.

The binding between $p21^{hRas}$ and compound **4**, one of the most active compounds in inhibiting nucleotide exchange in vitro, was investigated by STD NMR experiments in solution. The STD experiment (Figure 3) was carried out at 37 °C to overcome the solubility problems of compound **4** at room temperature in the 9% methanol/water mixture used for the experiment. The STD spectrum (Figure 3 d) clearly shows some aromatic signals of **4**. In agreement with observations on previously published Ras ligands,[8] the aromatic biphenyl groups appear to interact stronger with Ras than the tricyclic levoglucosenone-derived scaffold. This is further confirmed by the in silico binding mode.

To investigate a specific in vivo effect on Ras-mediated signaling, we tested the activity of the most active compounds **1** and **4**, on both normal cells and cells that had been transformed by the oncogenic mutant k-Ras G12R (Figure 4). Compound **4** partially inhibited the growth of normal NIH3T3 mouse fibroblasts at a concentration of 50 μM, whereas this effect was much more pronounced in k-Ras-transformed NIH3T3 cells, in which a complete inhibition of growth was observed. In contrast, compound **1** was nearly inactive toward both cell types.

Compounds **1**–**9** were tested for their inhibitory properties toward different cancer cells: the human ovarian cancer cell line A2780 and the human breast adenocarcinoma cell line MCF-7. Selected compounds were also tested on Ras-dependent cells, namely the human epithelial lung carcinoma cell line A549 and the colon cancer cell line HCT116. Compounds were tested up to a maximum concentration of 40 μM. IC_{50} values for the inhibition of cancer cell vitality are listed in Table 1.

Some members of our focused library of potential Ras inhibitors showed some interesting activities in vitro and toward cells. Compounds **1** and **4** were the most active in vitro in inhibiting the Cdc25-stimulated nucleotide exchange on Ras. In the case of compound **4**, Ras binding was also clearly observed in STD NMR experiments in solution. This compound also shows an interesting selectivity in inhibiting Ras-dependent growth and proliferation of k-Ras-transformed mouse fibroblasts. The selective toxicity shown by compound **4** on mutated versus normal cells is very promising for the development of agents that target oncogenic Ras mutants. This selectivity can be explained by the well-documented higher sensitivity of transformed cells toward certain types of signaling inhibitors.[16,3b] Compounds **3**–**8** are toxic to several tumor cell lines. The presence of the ketone group in compounds **1**–**5** could be responsible for decreased

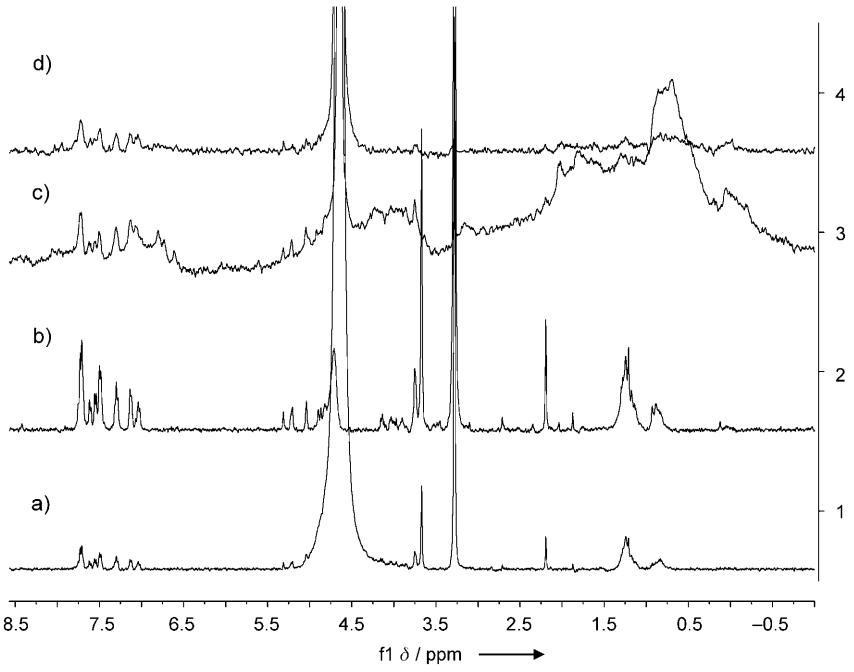


Figure 3. Compound **4** + Ras-GDP (30:1 in D_2O + 9% CD_3OD), 37 °C: a) ^1H, 128 scans; b) ^1H presaturated, 856 scans; c) STD, saturation frequency = 0.0 ppm, 3400 scans; d) STD with T_2 filter = 2 ms, saturation frequency = 0.0 ppm, 3400 scans.

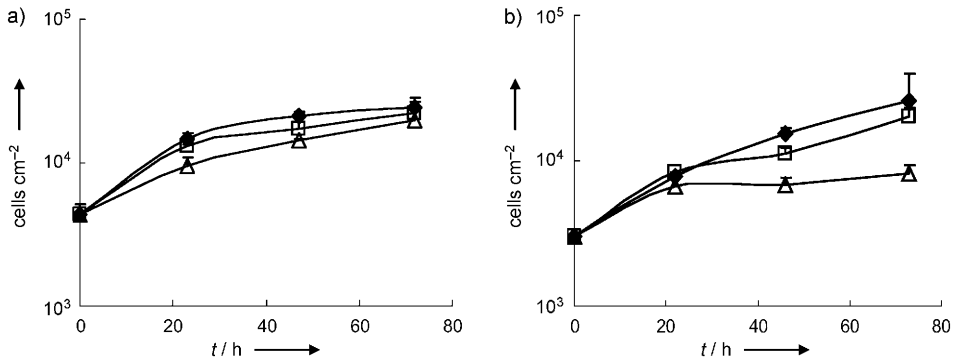


Figure 4. Mammalian cell growth inhibition: a) NIH3T3 and b) NIH3T3 k-Ras mouse fibroblasts were seeded into 60 mm dishes and grown for one day: four dishes were allowed to continue growing without addition of the inhibitor (◆), while compounds **1** (□), and **4** (△) were added to the other dishes at a final concentration of 50 μM. Sample cells were collected at various time points for determination of cell number. Standard errors were calculated on three independent experiments.

Table 1. Inhibition (IC_{50} [μM]) of cancer cell proliferation.

	Cell Line			
Compound	A2780	MCF-7	A549	HCT116
1	>40	>40	>40	>40
2	30	>40	NT[a]	NT
3	12	40	NT	NT
4	15	>40	>40	>40
5	16	>40	>40	>40
6	6.5	6.9	15.5	17
7	3.1	7.1	NT	NT
8	4.3	5.5	NT	NT
9	36	>40	>40	>40

[a] Not tested.

cell penetration and the cause of metabolic instability. The more active inhibitors of cell proliferation are compounds **6**, **7**, and **8**, probably because the presence of the benzyl ether at C4 increases lipophilicity and improves cell penetration. In conclusion, the combination of the levoglucosenone-derived tricyclic scaffold with biphenyl aromatic groups has led to the generation of new Ras inhibitors with interesting activity against a representative set of human cancer cell lines.

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Keywords: antitumor agents · cycloaddition · molecular modeling · NMR · signal transduction

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