ELSEVIER

Il Farmaco 53 (1998) 255–261

IL FARMACO

Modulation of glutamate receptor pathways in the search for new neuroprotective agents [1]

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Abstract

Excessive stimulation of excitatory amino acid (EAA) receptors is responsible for a wide variety of acute and chronic neurological impairments. A separate line of investigation has focused on oxidative stress as one of the main reasons for several of these degenerative disorders. Current evidence has confirmed that activation of both ionotropic and metabotropic glutamate receptors can also result in either neuroprotection or neurodegeneration according to the role played by oxidative stress mechanisms. An outline of this research, together with our recent results aimed at the discovery of new subtype selective modulators of the central nervous system pathways as well as new classes of free radical scavengers, is presented.

Keywords: Metabotropic glutamate receptors; Free radicals; 1-Aminoindan-1,5-dicarboxylic acid (AIDA); 2-(2′-Carboxy-3′-phenylcyclopropyl)glycine (PCCG) stereoisomers; Fullerene; Neuroprotective agents

1. Introduction

Neuronal death is the cellular end-point of a variety of both acute and degenerative diseases, including focal and global ischemia, hypoxia, traumatic brain injury, Parkinson's disease, Alzheimer's disease and Huntington's chorea. Whereas many individual mechanisms may be operative in triggering the neuronal injury, there is compelling evidence that two central events play a key role, namely, overstimulation of glutamate receptors (GluRs) which results in increased intracellular calcium ($[Ca^{2+}]_i$), and free radical injury [1]. Experimental evidence gathered over the last ten years has pointed out that these two events are strictly interconnected (see e.g. Refs. [2–4]). The transient overflow of Ca^{2+} which follows abnormal stimulation of glutamate receptors activates a number of intracellular mechanisms (among which are the activation of phospholipase A_2 and the opening of the mitochondrial permeability transition pores) that are potential sources of reactive oxygen species (ROSs) [5]. In turn, production of ROSs gives rise to a series of intracellular events such as lipid peroxidation, protein oxidation and protein cross-linking which result in cell death [6]. On the other hand, the abnormal ROS production that follows ischemia/reperfusion is by itself a cause of excessive release of glutamate into the synaptic cleft that in turn triggers the excitotoxic process. Following these observations, it has been proposed that both excitotoxicity and oxidative stress cooperate in a vicious cycle to the early onset and subsequent propagation of neuronal injury (Fig. 1) [7].

This hypothetical, and yet simplistic, cycle thus indicates that the still unachieved goal of pharmacological control of neurodegenerative diseases requires that new multiple and specific molecular targets have to be approached. Indeed, most of the strategies so far pursued towards neuroprotection against cerebral ischemia or neurodegenerative diseases have largely focused on modulation of postsynaptic glutamate receptors, either ionotropic or metabotropic. It is now becoming apparent, however, that only a combination of different modulators of several targets along the chain of events leading to neuronal death is suitable for clinical development and this opens new interesting perspectives for medicinal chemistry.

Being involved in the field of design and synthesis of neuroprotective agents for many years, we have dealt with several molecular targets, including ionotropic and metabotropic glutamate receptors (see e.g. Refs. [8–12]) and the kynurenine catabolic pathway of L-tryptophan (L-Trp) [13]. In this paper, we report some of our recent results obtained in the design and synthesis of subtype-selective metabotropic glutamate receptor agonists and antagonists as well as the synthesis and the possible biological implication of fullerene-based free radical scavengers.

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[1] Presented at the First Italian–Swiss Meeting on Medicinal Chemistry, Turin, Italy, September 1997.

0014-827X/98/$19.00
PII S0014-827X(98)00018-4

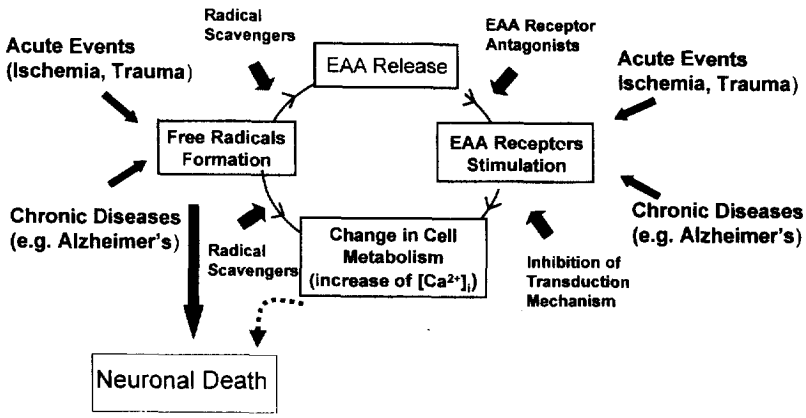


Fig. 1. Vicious cycle relating excitotoxic damage and free radical injury. Taken from Ref. [7].

2. Metabotropic glutamate receptors

The G-protein coupled family of metabotropic glutamate receptors (mGluRs) has been the object of intense studies that have permitted their molecular diversity, structure and physiopathological role to be elucidated. Metabotropic glutamate receptors are currently classified into three groups, according to their sequence homology, transduction mechanism and pharmacology (for recent reviews see Refs. [14–16]). Group I is constituted of mGluR1 and mGluR5 which are both coupled to the activity of phospholipase C (PLC) and intracellular calcium mobilization. Group I receptors are thought to be postsynaptically localized, even if a presynaptic localization has been suggested for one of them, regulating the glutamate release. Group II is formed by mGluR2 and mGluR3 which are negatively coupled to the activity of adenylyl cyclase and to voltage operated calcium channels. mGluR2 has a presynaptic localization, where it regulates the glutamate release, while mGluR3 is prevalently localized on astrocytes, being *N*-acetylaspartyl-glutamate (NAAG), a precursor of L-glutamic acid (L-Glu), the likely endogenous activator. Finally, group III is constituted of mGluR4, mGluR6, mGluR7 and mGluR8 which are negatively coupled to the activity of adenylyl cyclase. Physiologically regulating the slow synaptic transmission, mGluRs become involved in the fast transmission related to the entry of calcium into the neurons only in the case of synaptic hyperactivity through, for example, the activation of protein kinase C (PKC) or coupling to voltage operated cation channels. The intervention of mGluRs only in the case of glutamatergic stress makes their modulation an attractive target in the development of clinically useful drugs. Despite the variety of effects mediated by mGluRs, it is accepted as a rule of thumb that group I antagonists and group II/III agonists are expected to be endowed with neuroprotective activity [15]. Nevertheless, there is still a need for potent and subtype selective ligands, either agonists or antagonists, to be used for a more complete characterization of this family of receptors. Indeed, the growing knowledge on physiological aspects of mGluRs has not been accompanied for many years by a corresponding development of subtype-selective ligands.

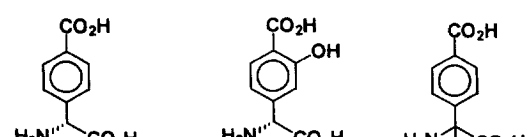
In general, almost all the mGluR ligands known so far belong to two classes of compounds, namely, the 2-(carboxycyclopropyl)glycines (CCGs) and the carboxyphenylglycines (CPGs). In this connection and as a continuation of our project aimed at the design and synthesis of conformationally constrained glutamate analogues, we have addressed ourselves to the task of defining those structural features of both classes of compounds which determine ligand binding and selectivity to individual mGluR subtypes.

2.1. Design and synthesis of new CPG derivatives

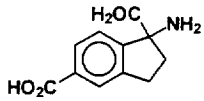
First reported as mGluR ligands by Watkins and coworkers in 1993 [17], carboxyphenylglycine derivatives display a wide range of activity depending on the type and degree of substitution on the aromatic ring. In particular, (*S*)-(4-carboxyphenyl)glycine (4CPG **1**), (*S*)-(4-carboxy-3-hydroxyphenyl)glycine (4C3HPG **2**) and (+)-α-methyl-(4-carboxyphenyl)glycine (M4CPG **3**) (Scheme 1) have shown interesting properties as mGluR1 antagonists, albeit with some activity as mGluR2 agonists [18,19].

With the aim of disclosing which structural features of CPGs are relevant in determining mGluR1 potency and selectivity we have investigated the effect of structural manipulation of (i) the side chain and (ii) the aromatic ring of CPGs.

CPGs have the possibility of freely rotating around the Cα–C1 bond, and it is expected that the different activities of representative members of CPGs at mGluR1 or mGluR2 is a consequence of this conformational flexibility. The insertion of the 4CPG **1** core into a rigidified bicyclic structure



Scheme 1. (Carboxyphenyl)glycine (CPG) derivatives as metabotropic glutamate receptor ligands.



AIDA

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Scheme 2. (±)-1-Aminoindan-1,5-dicarboxylic acid (AIDA).

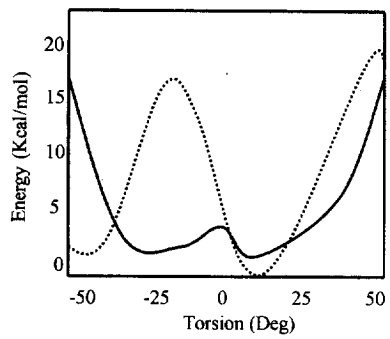


Fig. 2. Potential surface of AIDA (bold line) compared with that of 4CPG (thick line).

yielded (±)-1-aminoindan-1,5-dicarboxylic acid (AIDA **4**) [20] (Scheme 2).

Molecular modelling studies have demonstrated that AIDA **4** can exist in two conformational states, corresponding to the envelope extremes of the 5-membered ring. When compared with the potential surface of 4CPG **1**, it became apparent that AIDA **4** is able to mimic only one of the conformational minima of **1** (Fig. 2), which can therefore be considered the bioactive conformation of CPGs when acting at mGluR1 receptor subtypes.

When tested on BHK cells individually expressing mGluR1, mGluR2 or mGluR4, AIDA **4** was shown to be a selective mGluR1 antagonist ($IC_{50}=214$ μM), albeit with a reduced potency with respect to the parent CPG derivatives, with no activity at either mGluR2 or mGluR4 [20]. Furthermore, when tested at concentrations up to 1 mM, AIDA **4** inhibited the ACPD-induced release of aspartate from in vivo rat cortex, thus suggesting a potential presynaptic localization of mGluR1 [21]. Also, administered at 100 nM doses, AIDA showed full neuroprotection in the CA1 model of global ischemia in gerbils, thus demonstrating that mGluR1 antagonists are endowed in vivo with neuroprotective properties [22]. The biological characterization of AIDA **4** in cellular lines expressing individual mGluR subtypes has, moreover, demonstrated that conformational constraint of CPGs into more rigid structures is able to confer subtype selectivity, a property that can be instrumental in the design of new mGluR1-selective ligands.

A second characteristic of all the CPG derivatives is the collinearity between the α-amino acidic moiety and the ω-carboxylate group. This characteristic is conferred by the presence of the phenyl ring and it is generally accepted as a crucial feature of mGluR1 antagonists. There is the possibility, however, that the phenyl ring not only serves as a suitable spacer between pharmacophoric groups but also plays a specific role in the ligand–receptor interaction. In order to clarify this point we have designed and synthesized (*S*)-(+)- and (*R*)-(−)-2-(3′-carboxybicyclo[1.1.1]pentyl)glycine ((*R*)-CBPG **5** and (*S*)-CBPG **6**) [23] (Scheme 3).

R-CBPG

5

S-CBPG

6

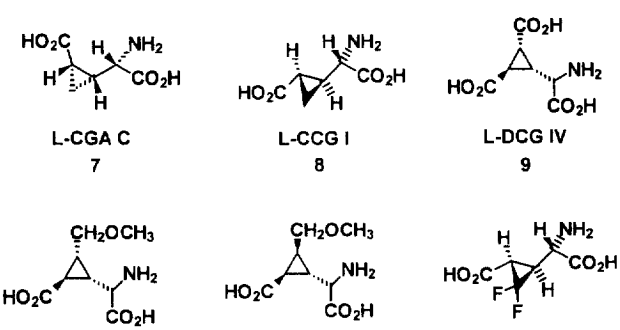
Scheme 3. 2-(3′-Carboxybyciclo[1.1.1]pentyl)glycine (CBPG) derivatives.

In these compounds, the bicyclo[1.1.1]pentane moiety is still able to keep the α-amino acidic moiety and the ω-carboxylate group in a collinear disposition, but is endowed with quite a different stereo-electronic profile with respect to the phenyl ring.

When evaluated on BHK cellular lines individually expressing mGluR1, mGluR2, mGluR4 or mGluR5, (*R*)-CBPG **5** was completely inactive, whereas (*S*)-CBPG **6** was shown to be a potent ($IC_{50}=25$ μM) and rather selective mGluR1 antagonist, with no effect at group II or III mGluRs; (*S*)-CBPG is, however, a weak partial agonist at mGluR5 with $IC_{50}=103$ μM [23]. Also, (*S*)-CBPG **6** was shown to be endowed with neuroprotective activity when administered at doses up to 100 nM in the CA1 model of global ischemia in gerbils. These data confirm the hypothesis that the presence of an aromatic ring is not required for mGluR1 antagonist activity provided that a suitable spacer is able to keep the pharmacophoric groups in a collinear disposition. Studies are in progress to evaluate whether larger cyclic systems are able or not to fit the receptor site cavity and the results will be reported in due course.

2.2. Design and synthesis of new CCG derivatives

Carboxycyclopropylglycines (CCGs) (Scheme 4) have represented a valuable source of partially constrained glutamate analogues and have been demonstrated to be useful tools for probing the conformational requirements of excitatory amino acid receptors. Indeed, the introduction of the cyclopropyl moiety into the glutamate skeleton reduces to a great extent its conformational flexibility, allowing selective inter-



L-CGA C

7

L-CCG I

8

L-DCG IV

9

c-MCG I

10

t-MCG I

11

L-F2-CG I

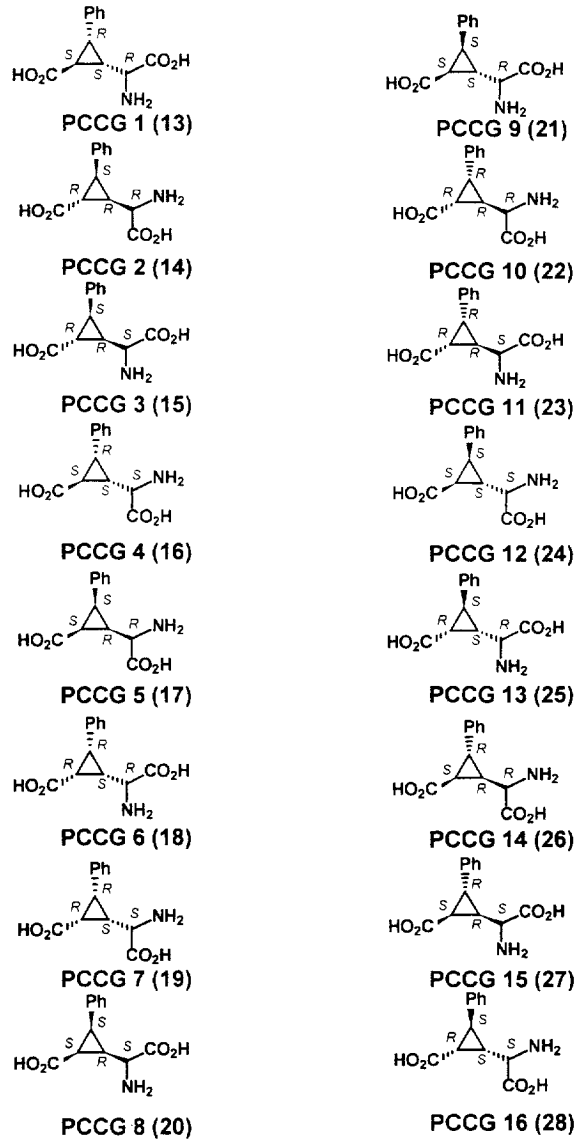
12

Scheme 4. (Carboxycyclopropyl)glycine derivatives as glutamate receptor ligands.

action with a number of recognition sites, including ionotropic NMDA receptors, metabotropic receptors and uptake proteins.

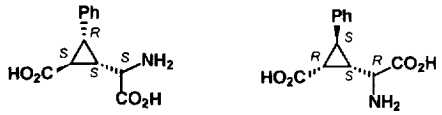
Indeed, we first reported that among the eight possible isomers of CCGs, (2*S*, 1′*R*, 2′*S*)-CCG (CGA C **7**) is a potent and selective NMDA agonist [24]. In 1989, Shinozaki's group identified the (2*S*, 1*S*′, 2′*S*)-CCG isomer (L-CCG I **8**) as a potent and rather selective group II mGluR agonist [25]. Since then, L-CCG I **8** has been widely used either in molecular modelling studies [26] or as a template for further chemical manipulation in the search for more potent and selective mGluR ligands. A successful strategy was the introduction of different substituents in position 3′ of the cyclopropyl ring. Thus, the dicarboxy derivative (DCG IV **9**) was reported to have an improved potency and selectivity with respect to the parent derivative as a group II mGluR agonist, albeit endowed with some NMDA receptor activity at high doses [27,28]. Following this strategy, other substituents have successfully been introduced in position 3′, including the methoxymethyl group (cMCG I **10** and tMCG I **11**) [29], and the difluoro substituent (2F-CG I **12**) [30]. In view of the interesting pharmacological profile displayed by trisubstituted CCGs, we have undertaken a research project aimed at investigating the still unexplored effect of introducing a bulk, hydrophobic substituent in position 3′. We thought that a phenyl ring could prove useful for probing the steric accessibility of still unexplored areas of receptor sites of different members of the glutamate receptor family. The introduction of a substituent in position 3′ generates one more chiral centre with respect to parent CCGs, so that 16 diastereoisomers are expected. The availability of such a stereo library of diastereoisomers, each one encoding a particular conformation of glutamic acid, might be useful for the better definition of already known members and for the characterization of still unknown members of the glutamate receptor family. Accordingly, we have envisaged an enantiodivergent synthetic protocol for the synthesis of the 16 2-(2′-carboxy-3′-phenylcyclopropyl)glycine (PCCG) stereoisomers **13–28** (Scheme 5) whose key step is the diastereoselective Streker reaction involving the nucleophilic addition of a cyanide ion to Schiff bases formed by condensation of suitable racemic aldehydes with optically active α-phenylglycinol. Four diastereoisomeric α-aminonitrile derivatives are expected to form from each aldehyde; the subsequent oxidative cleavage followed by acidic hydrolysis and ion-exchange chromatography afforded the 16 derivatives **13–28** [31].

The stereo library thus obtained was submitted to a variety of biological assays, including (i) inhibition of binding of selective ligands for AMPA, NMDA and KA receptors from rat membranes, (ii) inhibition of binding of labelled glutamate to membranes prepared from BHK cells expressing mGluR1a, or of binding of labelled L-2-amino-4-phosphonobutyrate to membranes expressing mGluR4, (iii) inhibition of Na^+-dependent glutamate transport into synaptosomes and of Ca^{2+}/Cl^--dependent glutamate uptake, (iv) antagonism of the effects of glutamate on PI hydrolysis or forskolin-stimulated cAMP formation in BHK cells expressing mGluR1, mGluR2 or mGluR4, (v) inhibition or stimulation of the glutamate-induced activity of phospholipase D (PLD). Some of the PCCG stereoisomers were shown to be able to displace selective ligands from ionotropic receptors, whereas others were able to significantly inhibit Ca^{2+}/Cl^--dependent glutamate uptake. PCCG 12 **24**, in particular, is the most potent inhibitor of this class so far reported, with $IC_{50} = 7$ μM. The two most interesting compounds are, however, PCCG 4 **16** and PCCG 13 **25** (Scheme 6).



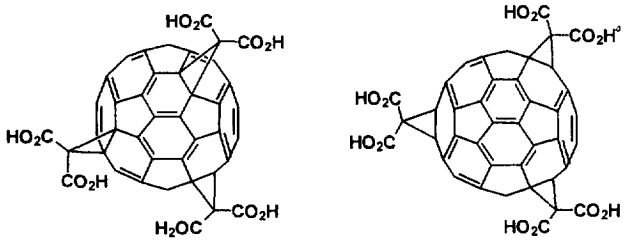
Scheme 5. 2-(2′-Carboxy-3′-phenylcyclopropyl)glycines (PCCGs): a stereo library.

The latter is the first selective and very potent ($IC_{50} = 90$ nM) antagonist of the newly pharmacologically characterized metabotropic glutamate receptor coupled to the activity of PLD [32,33]. The availability of such a potent and selective antagonist will certainly be of help in better characterizing this new member of the mGluRs. PCCG 4 **16**, on the other hand, is a potent ($IC_{50} = 8$ μM) and selective antagonist



PCCG 4 (16) PCCG 13 (25)

Scheme 6. PCCG 4 **16** and PCCG 13 **25**, potent and selective antagonists at group II and PLD coupled mGluRs, respectively.



Scheme 7. Tris-dicarboxymethanofullerene derivatives **30** and **31**.

of mGluR2 receptor subtype, being also a partial agonist at mGluR4 at higher doses (EC_{50} = 156 μM). In light of the pharmacological relevance of PCCG 4 **13**, we have devised its enantioselective synthesis [34]. Efficient access to optically pure PCCG 4 **13** will be useful for understanding the physiopathological role of group II mGluRs [35].

3. Fullerene-based free radical scavengers

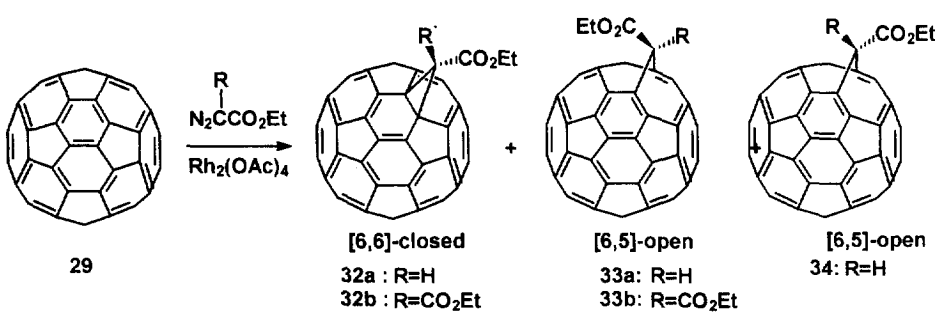
The availability of macroscopic quantities of C60-fullerene **29**, the third allotropic form of carbon, has stimulated intense research activity directed to the understanding of its physicochemical behaviour and its reactivity.

Indeed, an impressive amount of work has been accumulated over the last years on the chemistry of C60-fullerene, going from basic theoretical studies to possible application to material and biomedical sciences [36]. These achievements have constantly been flanked by hypotheses on possible biological applications of fullerene derivatives or C60-fullerene itself. Indeed, a variety of biological actions of fullerene derivatives have been reported, including antiviral activity, enzyme inhibition activity, and cytotoxic activity after irradiation [37]. Although many of the early promises of C60-fullerene in this field seem not to have been kept, renewed interest comes from the observation that C60-fullerene may act as a powerful radical scavenger and thus could be employed as an antioxidant in certain neurological diseases. The potential of C60-fullerene as a radical scavenger derives from its electronic structure as an electron-poor polyolefine with triply degenerated lowest unoccupied molecular orbitals (LUMOs), able to accept up to six electrons. It has been demonstrated, in this context, that C60 readily accepts up to 34 methyl [38] or 20 hydroxy radicals [39], thus forming polymethylated or polyhydroxylated fullerene derivatives, a property that can usefully be instrumental in designing biological free-radical scavengers, provided that the fullerene derivative is able to reach the functional district of action. In a recent report, Choi et al. have reported that the two water-soluble tris-dicarboxymethanofullerene derivatives endowed with C_3 (**30**) or D_3 (**31**) symmetry (Scheme 7) showed interesting neuroprotective properties when tested against NMDA- or AMPA-induced excitotoxicity or glucose deprivation [40] .

The absence of direct interaction with glutamate receptors suggests that the neuroprotective activity of **30** and **31** derives from their radical scavenger properties, as confirmed by electron paramagnetic resonance (EPR) spectroscopy. Motivated by these results, we have undertaken a research project directed to the development of new synthetic methodologies aimed at the functionalization of C60-fullerene **29** towards water-soluble derivatives. So far, alkoxymethanofullerenes have been prepared either by nucleophilic attack of bromodiethylmalonate in the presence of bases [41] or by thermal decomposition of α-diazoester compounds followed by addition to C60 of the resulting carboalkoxycarbenoids [42,43]. As an alternative synthetic strategy, we have directed our attention to the still unreported reaction of C60-fullerene **29** with carboalkoxycarbenoids generated by dirhodium(II) tetraacetate-mediated decomposition of diazocarbonyl precursors such as ethyldiazoacetate (EDA) and ethyldiazomalonate (EDM) (Scheme 8) [44].

The reaction of C60 **29** with EDA in the presence of dirhodium(II) tetraacetate was investigated first under different conditions and the results are reported in Table 1.

We found that the best yield was achieved when a stoichiometric amount of dirhodium(II) tetraacetate in α-methylnaphthalene was used, whereas other solvents or a catalytic amount of dirhodium(II) tetraacetate dramatically decreased



Scheme 8. Reaction of C60-fullerene **29** with carboxyalkoxycarbenoids.

Table 1
Comparison between thermal and rhodium(II) tetraacetate-mediated decomposition of EDA and EDM in the presence of fullerene

Conditions	R	Solvent	T (°C)	t (h)	Yield (%)	Ratio of formation		
						32	**33**	**34**
Thermal [a]	H	PhMe	110	7	35	1	4	2
Rh-stoichiom.	H	PhMe	r.t.	20	21	14	1	1
Rh-stoichiom.	H	1-Me-Naphth.	r.t.	8	42	52	1	–
Thermal [b]	CO_2Et	PhMe	110	20	10	N.D. [c]	N.D. [c]	N.D. [c]
Rh-stoichiom.	CO_2Et	1-Me-Naphth.	80	32	33	9	1	–

[a] See Ref. [43].
[b] See Ref. [45].
[c] Not determined.

the yield of conversion. Compared with the thermal decomposition of EDA, the dirhodium(II) tetraacetate-mediated reaction proceeds under much milder conditions in higher yield and, most interestingly, with high selectivity of the [6,6]-closed methanofullerene (**32a**) product over [5,6]-open fulleroid derivatives **33a**, **34**. The same results were obtained when the less reactive EDM was employed. Compared with the thermal decomposition [45], the dirhodium(II) tetraacetate-mediated reaction of EDM with fullerene required milder conditions (80°C versus refluxing toluene) and gave higher yield (33% versus 10%) of [6,6]-closed biscarboxymethanofullerene **32a** as the only reaction product.

Computational studies are in progress aimed at understanding the mechanistic details of the dirhodium(II) tetraacetate-mediated addition of carboalkoxycarbenoids. In particular, the following points have to be addressed: (i) the requirement of a stoichiometric instead of catalytic amount of dirhodium(II) tetraacetate; (ii) the pathway leading to the formation of the methanofullerene product (i.e. nucleophilic attack of the rhodium atom on the 6,6-double bond or nucleophilic attack of the 6,6-double bond on the carbenoidic carbon atom); (iii) the high chemoselectivity between methanofullerene and fulleroid isomers. In any case, in view of the mildness and the high specificity, the transition metal-mediated carbenoid addition of α-diazoesters to fullerene can be employed in the selective synthesis of carboalkoxy substituted [6,6]-methanofullerene. Biological studies are also in progress to verify the effectiveness of derivative **32** as a free radical scavenger in a number of in vitro assays.

4. Conclusions

The continuous progress in the characterization of the molecular mechanisms that trigger neuronal injury offers new targets for medicinal chemists. The design and synthesis of selective ligands acting along the chain of events leading to neuronal death, from membrane receptors to intracellular effector systems, will be of help in understanding the specific role played by each system and in the development of clinically useful neuroprotective agents.

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