

Identification of GCP II Inhibitors Based on 4-Arylmethyl-3-(4-carboxyphenyl)-5-hydroxyisoxazole Scaffold

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Received September 01, 2008; Revised October 07, 2008; Accepted October 08, 2008

Abstract: Screening of a non-ionizable compound library and hit optimization studies has resulted in a series of compounds based on a 4-arylmethyl-3-(4-carboxyphenyl)-5-hydroxyisoxazole scaffold with GCP II inhibitory activity in the low micromolar range.

Keywords: Glutamate carboxypeptidase II, Glutamate, 5-hydroxyisoxazole, Lipophilicity, Synthesis.

INTRODUCTION

The modulation of excitotoxic glutamate release is under investigation as a therapeutic target in several neurological disorders. Among others, decreasing the concentration of glutamate and increasing the concentration of neuropeptide *N*-acetylaspartylglutamate (NAAG) by inhibition of glutamate carboxypeptidase II (GCP II) has attracted attention as a potential approach to achieve neuroprotection under neuropathological conditions associated with dysfunction of the glutamatergic system [1-4]. GCP II is a membrane bound Zn²⁺ metallopeptidase that cleaves the endogenous (NAAG) producing *N*-acetylaspartate and glutamate [5, 6]. The endogenous substrate of this enzyme, NAAG, acts as an agonist of mGluR3 receptors, activation of which inhibits pre-synaptic release of glutamate and stimulates the release of neurotrophic factors [7, 8]. A number of competitive inhibitors (Fig. 1, representative examples 1-4) have been discov-

inhibitor 3 exhibited good oral availability and tolerance in phase I clinical trials [3, 4, 14].

Known GCP II inhibitors typically consist of a glutamate fragment which is linked to the Zn²⁺ binding group. Recently, structures incorporating a carboxyphenylacetic acid fragment were also found to be potent inhibitors (for example compound 4) [16]. The very polar nature of these polycarboxylic acid derivatives prevents their passive penetration through the blood-brain barrier (BBB) under physiological conditions [17]. The development of more lipophilic inhibitors would be desirable and several attempts in this direction have been made by reducing the number of fully ionizable groups in the molecule [12, 14-16].

Our research project aimed to find GCP II inhibitors with increased lipophilicity that could facilitate their BBB penetration by passive transport.

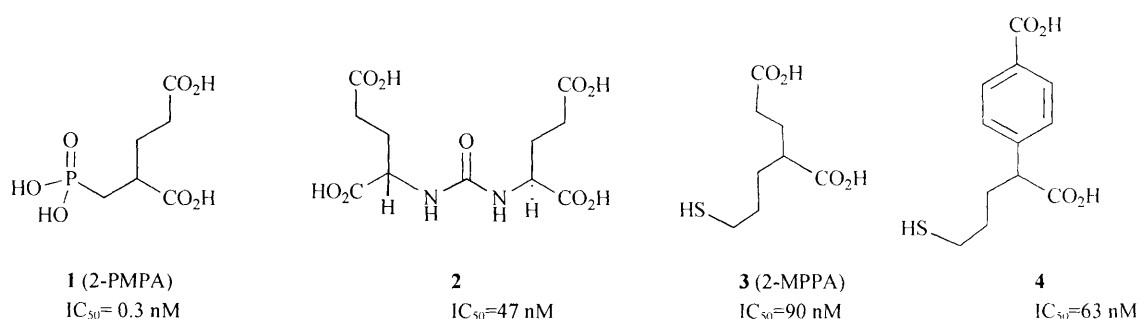


Fig. (1) Representative examples of GCP II inhibitors.

ered, the most potent of which exhibited GCP II inhibitory activity in the nanomolar range [1, 3, 9-16]. Several compounds have been shown to be effective in animal models of chronic and acute neurological disorders [1-4]. Moreover,

At an early stage of the project, the screening of a non-ionizable compound library was performed, and this resulted in a hit 5 with GCP II inhibitory activity IC₅₀ < 10 μM (18% of control at 10 μM) (Fig. 2) [18]. Compound 5 displayed low stability in buffer, likely due to the presence of an activated double bond, which prohibited further optimization of this scaffold. In this paper we report the development of the hit 5 to relatively stable 5-hydroxyisoxazole derivatives with general structures 6 and 7, representatives of which showed low micromolar GCP II inhibitory activity.

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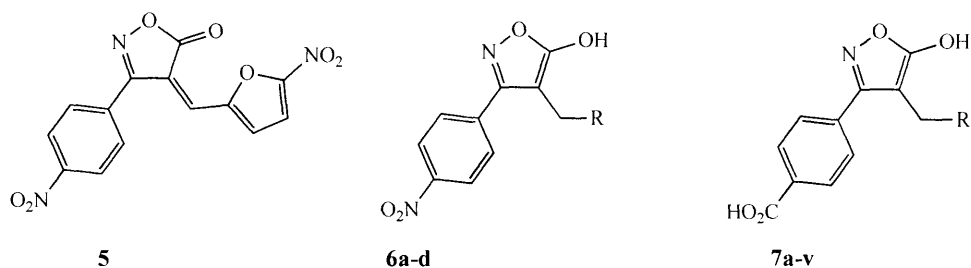


Fig. (2). Structures of screening hit **5** and 5-hydroxyisoxazoles **6,7** resulting from hit optimization studies.

CHEMISTRY

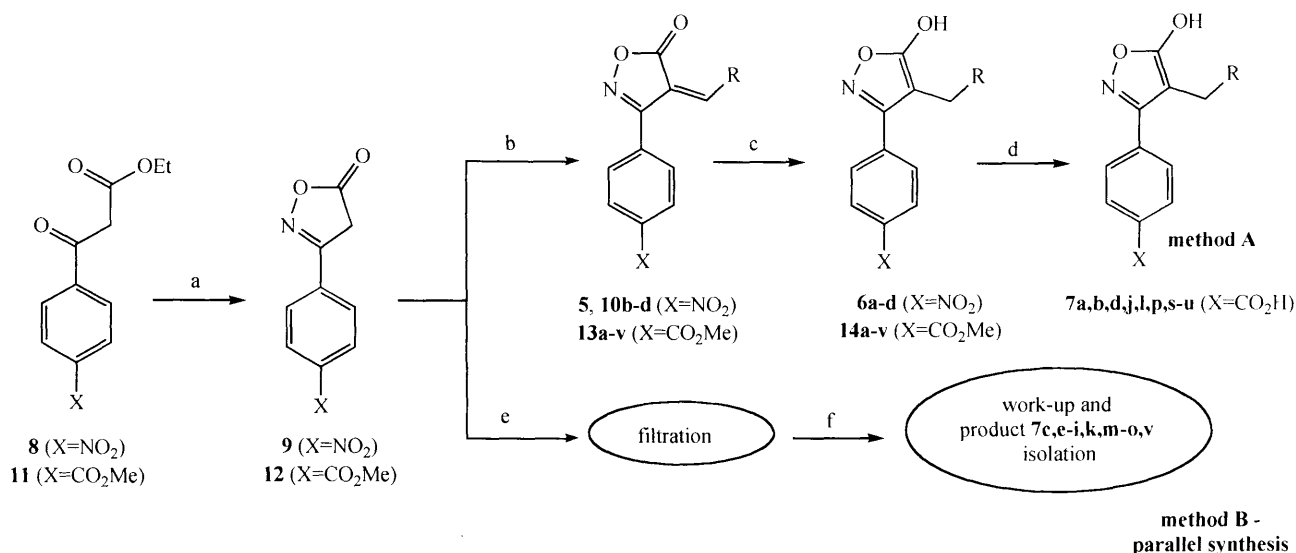
The synthesis of compounds **6a-d** started from β -ketoester **8** that, following the reaction with hydroxylamine, gave the isoxazolone derivative **9** [19]. This was condensed with a range of commercial aldehydes to give arylmethylideneisoxazolone derivatives **5** and **10b-d** [19]. The reduction of the double bond resulted in 4-substituted 3-(4-nitrophenyl)-5-hydroxyisoxazoles **6a-d** (Scheme 1, Fig. 2, Table 1) [19]. Carboxylic acid analogues **7a-v** were prepared in a similar way (Scheme 1, Fig. 2, Table 1). The key building block **12** was obtained from β -ketoester **11**. Two methods for the preparation of the desired 5-hydroxyisoxazoles **7a-v** from **12** were developed. A conventional method (method A) followed the manner of preparation of compounds **6a-d** with one additional hydrolysis step. Building block **12** was first condensed with commercial aldehydes, and then the double bond reduction with NaBH_4 and methyl ester hydrolysis under basic conditions gave 4-substituted 3-(4-carboxyphenyl)-5-hydroxyisoxazoles **7**. As a parallel synthesis approach, we have developed a more convenient procedure in which all three last synthetic steps were combined (method B). Condensation product **13** was filtered off, and without drying, it was reduced with NaBH_4 . After the reduction was complete, hydrolysis was performed in the same reaction vessel. In the majority of cases, this allowed an increase of the overall yield of the product **7**.

RESULTS AND DISCUSSION

The 5-hydroxyisoxazole analogue **6a** of compound **5** displayed detectable GCP II inhibitory activity at $10 \mu\text{M}$. However, the replacement of the 5-nitrofuryl group by 3-fluoro-, 3-chloro- or 3-nitrophenyl groups resulted in practically inactive compounds **6b-d**.

The hydroxyisoxazole containing nitrofuryl moiety **7a** displayed notable GCP II inhibitory activity, and in contrast to its analogue **6a**, the replacement of the nitrofuryl moiety with phenyl- or substituted phenyl group was possible without significant loss of activity - several compounds like **7c-j** showed GCPII inhibitory activity in low micromolar range ($\text{IC}_{50} = 1-5 \mu\text{M}$). Further alterations of the substituent position 4 of the isoxazole led either to similar or lower GCP II inhibitory activity (compounds **7k-v**).

The structure of the newly found GCP II inhibitors **7** contain only one carboxylic group plus a 5-hydroxyisoxazole moiety with a $\text{pK}_a \sim 7$ which could be considered to be a partially ionisable carboxylic acid bioisostere [20]. The 4-carboxyphenyl-5-hydroxyisoxazole part of compounds **7** shares structural similarities with the P1' side chain of the known inhibitor **4** and thus likely interacts with the S1' pocket of GCP II. It is noteworthy that compounds having bulky substituents at position 4 of the isoxazole, for example compounds **7f-j**, have activity better than that of unsubstituted analogue **7r**. The most interesting in the series is com-



Scheme 1. Reagents and conditions: (a) $\text{NH}_2\text{OH}\cdot\text{HCl}$, MeOH, reflux; (b) RCHO, EtOH, reflux (c) NaBH_4 , EtOH, rt; (d) only for the synthesis of **7a-v**; NaOH, THF/H₂O, rt; (e) RCHO, EtOH, 55 °C; (f) NaBH_4 , EtOH, rt, after 1h NaOH.

Table 1. Substitution Pattern and GCP II Inhibitory Activity of 5-Hydroxyisoxazole Derivatives 6 and 7 [18]

No	X	R	% of Ctrl at 10 μ M	IC ₅₀ , μ M	Synt. Method
6a	NO ₂	5-(NO ₂)-2-furyl	54.8	18.9	A
6b	NO ₂	3-(F)Ph	69.2	-	A
6c	NO ₂	3-(Cl)Ph	82.2	-	A
6d	NO ₂	3-(NO ₂)Ph	94.4	-	A
7a	CO ₂ H	5-(NO ₂)-2-furyl	26.7	-	A
7b	CO ₂ H	3-(HO ₂ C)Ph	16.2	0.8	A
7c	CO ₂ H	3,5-Di-(<i>t</i> -Bu)-2-(HO)Ph	28.5	2.5	B
7d	CO ₂ H	3-(F)Ph	30.6	2.4	A
7e	CO ₂ H	1H-Indol-3-yl	31.6	2.3	B
7f	CO ₂ H	3-(PhCH ₂ O)Ph	31.7	3.2	B
7g	CO ₂ H	4-(PhCH ₂ O)Ph	33.1	3.4	B
7h	CO ₂ H	4-Br(Ph)	34.3	2.7	A
7i	CO ₂ H	3,5-Di-(<i>t</i> -Bu)Ph	36	3.3	B
7j	CO ₂ H	3-Br(Ph)	40.4	5.1 ^a	A
7k	CO ₂ H	Naphthalen-2-yl	44.0	-	B
7l	CO ₂ H	3-(Cl)Ph	47.0	-	A
7m	CO ₂ H	9H-Fluoren-2-yl	47.1	-	B
7n	CO ₂ H	4-Biphenyl-4-yl	47.1	-	B
7o	CO ₂ H	3,5-Di-(<i>t</i> -Bu)-4-(HO)Ph	47.1	-	B
7p	CO ₂ H	3-(HO)Ph	50.3	11.8 ^d	A
7r	CO ₂ H	Ph	54.2	23.0 ^d	B
7s	CO ₂ H	3-(NO ₂)Ph	57.8	-	A
7t	CO ₂ H	3-(CF ₃)Ph	59.0	-	A
7u	CO ₂ H	3-(HO)-4-(MeO)Ph	76.4	-	A
7v	CO ₂ H	(<i>Z</i>)-PhCH=CH	84.2	-	B

^aIC₅₀ is an estimate from GCP II inhibition at two concentrations (10⁻⁵M and 10⁻⁶M).

compound 7i (R= 3,5-di-(*t*-Bu)Ph) which shows low micromolar activity and has considerably increased lipophilicity compared to other members of series. The tolerance of such a bulky group 3,5-di-(*t*-Bu)Ph implies that the substituent at position 4 of the isoxazole lies outside the rather narrow S1' binding pocket [21] and could be used to improve the overall lipophilicity as well as slightly enhance activity. In addition, disposition of this part of the molecule outside the binding cleft may explain markedly flat SAR of compounds 7a-v.

In summary, we have identified a series of compounds based on a 4-arylmethyl-3-(4-carboxyphenyl)-5-hydroxyisoxazole scaffold with GCP II inhibitory activity in the low micromolar range. Our current efforts are directed to the development of GCP II inhibitors with nanomolar activity based on the newly found scaffold by using the information obtained from molecular docking of the most active compounds 7a-j into the active site of GCP II.

ACKNOWLEDGEMENTS

The work was partially supported by the European Social Fund within the National Programme "Support for Carrying

out Doctoral Study Programs and Post-Doctoral Researches" and Taiho Foundation of Latvia.

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- [18] The GCP II inhibitory activity of compounds 5-7 was quantified by assessing the rate of hydrolysis of [3 H]-NAAG (NEN Life Science Products) as previously described.⁵⁰ Briefly, the total reaction volume of 500 μ l contained rat forebrain membrane protein 20-40 μ g; Tris-HCl 50 mM containing 1 mM ZnCl₂, pH 7.4; 4 nM [3 H]-NAAG; inhibitor 10 μ M (for IC₅₀ 7 point DRC); 37°C; 40-45 min. The reactions were terminated by adding ice cold phosphate buffer (100 mM, pH 7.4). Nonspecific/background activity was determined in the presence of 10 μ M 2-PMPA. [3 H]-Glutamate was separated from residual [3 H]-NAAG on the Dowex AG 1-X8 anion exchange resin. After that the scintillation cocktail OptiPhase "Supermix" (Perkin Elmer) was added to the respective fractions and the elute radioactivity was determined by scintillation spectrometry [1, 9].
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