# Synthesis and Biological Activity of New Mixed HIV-PR Inhibitors Conjugated to Bifunctional High-Molecular Weight Poly(Ethylene Glycol)

Fabio Benedetti, Federico Berti, Gian M. Bonora, Pietro Campaner and Sara Drioli*

*Department of Chemical Sciences, University of Trieste, via Giorgieri 1, 34127 Trieste, Italy*

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**Abstract:** We have synthesized and evaluated a small set of dual action HIV-Protease inhibitors, consisting of a catalytic site reversible inhibitor conjugated with a dimerization peptidic inhibitor, linked *via* a suitable selective end-modified poly(ethylene glycol), in an effort to obtain a synergistic effect with improved biological behaviour and more powerful pharmacological features, such as water solubility, cell permeability and bioavailability.

**Keywords:** Poly(ethylene glycol), HIV-PR inhibitors , mixed conjugates.

## INTRODUCTION

HIV-1 protease (HIV-1 PR) is an attractive target for the design of inhibitors capable of effective antiretroviral therapy. Many potent protease inhibitors that bind inside the catalytic site of HIV-1 PR have been developed and several are used in combination therapies for AIDS. The main characteristic of such compounds is the presence of a dipeptide isostere that mimics the transition state of an amide bond hydrolysis. However, the rapid occurrence of resistance against these inhibitors due to the mutation of HIV-1 PR during clinical treatments is one of the major problems. It is thus, important to develop new protease inhibitors that are less sensitive to mutation causing drug resistance.

Dimerization of HIV-1 PR is another of the essential events to attain the mature structure, that is an enzymatically active C2-symmetric homodimer. The amino acid residues involved in the interfacial region for the dimerization of the HIV-1 PR monomer are conserved [1] in numerous resistant strains, and for this reason the inhibitors targeted to the dimerization interface may effectively function against the mutants as well as native HIV-1 PR. In addition, such dimerization inhibitors may decrease the occurrence of mutation itself during long-term clinical treatment. Oligopeptides truncated or acetylated at the amino terminal, containing the same amino acidic residues of the N- and C-terminal sequences present in the dimerization site, have proven to be effective as dimerization inhibitors [2, 3]. This finding has lead low molecular weight pseudopeptide inhibitors, characterized by the presence of functionalized aromatic groups [4], non-natural amino acids [5] or small peptides, where the amidic nitrogens are alkylated [6]. Such inhibitors cause the irreversible inactivation of HIV-1 PR [7], but their activity is generally moderate, with $IC_{50}$ values in the micromolar range [8].

*Address correspondence to this author at the Department of Chemical Sciences, University of Trieste, via Giorgieri 1, 34127 Trieste, Italy; Tel: +39 040 5583974; Fax: +39 040 5583903; E-mail: sdrioli@units.it

## RESULTS AND DISCUSSION

On these basis, we have designed a novel class of inhibitors, containing both a catalytic site and a dimerization inhibitor linked together by a suitable spacer as a poly(ehtylene glycol) chain, in order to obtain a synergistic, dual inactivation of the proteolytic activity.

A similar approach has already been reported in literature [9]. This is based on the conjugation of two different anti-HIV drugs on the same soluble support, characterized by bonds easily *in vivo* hydrolizable, thus giving the two free active inhibitors. In that case, however, the inhibitors used are active on two different target enzymes (HIV-PR and HIV-RT); instead our aim is to obtain conjugates that bear two different inhibitors active on the same target, but with different, possibly synergic, mechanisms and, containing a sequence also recognized by mutated HIV-PR, active against viral mutants.

The active molecules were selected on the basis of their known properties. Compounds **1a** and **2a** (Fig. **1**) are catalytic site inhibitors based on already reported Phe-Phe [10] and Phe-Pro [11] dihydroxyethylene pseudopeptides, while **3a** is a commercial inhibitor currently used in clinical AIDS treatment (Nelfinavir or Viracept®); their activity lies in the nano- to micromolar range (Table **1**). Nelfinavir **3a** was extracted and purified from Viracept capsulae, and then reacted at the phenolic function with bromoacetate to give compound **3b** which contains the free carboxylic group demanded for the final conjugation step.

Peptide **4** has been proposed as a dimerization inhibitor with a moderate activity, while 5 and 6 are the shortest peptide chains recognized at the dimerization site level. These three simple peptides were synthesized following the same standard protocol for peptide coupling in solution.

The core isostere **7** (Fig. **2**) has been synthesized following a known protocol [12], and then coupled to kynurenic acid at its free amino terminal, deprotected at the Boc-amino group and finally coupled also on this side with N-Boc-Trp-Ser-OH to give compound 1b, suitable for further elaboration at its Boc-protected terminus. Compound

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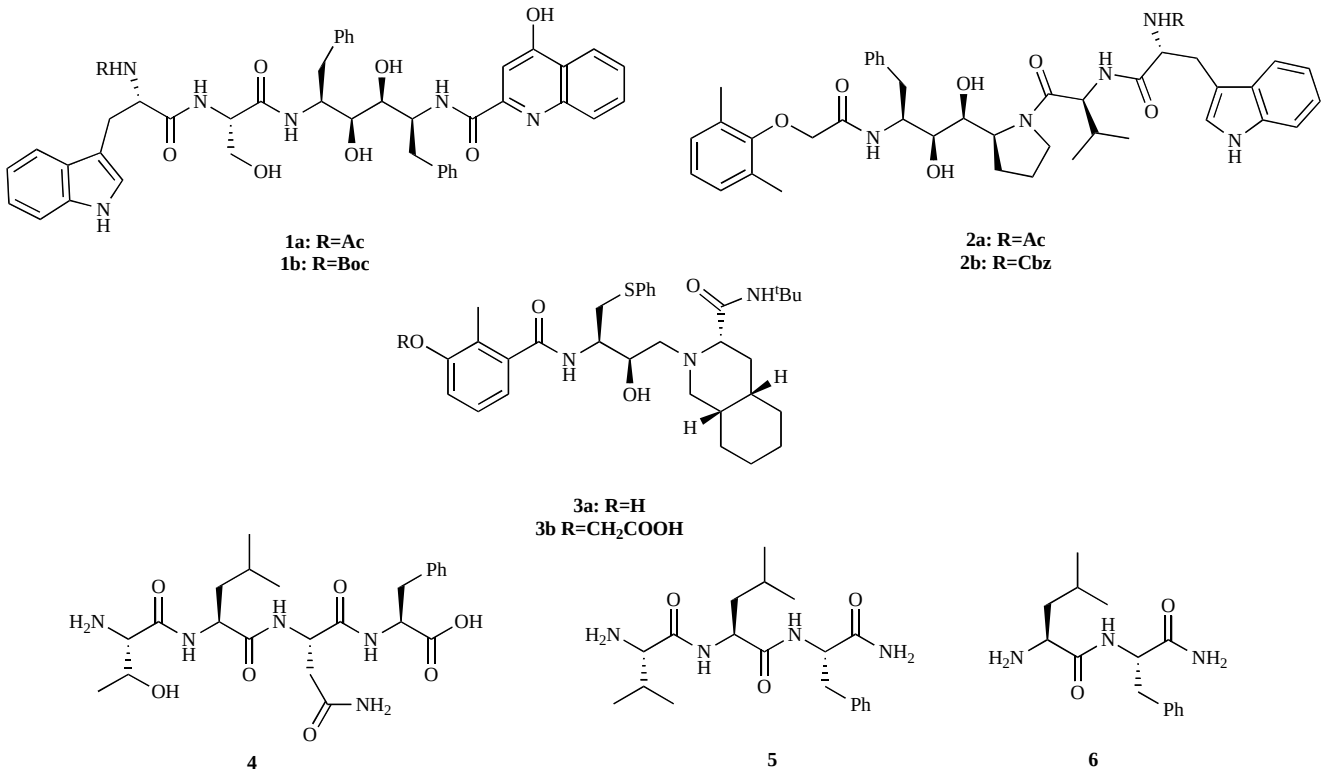



**Fig. (1).** Selected catalytic site and dimerization site inhibitors

2b was obtained in a similar way from the core structure 8 [13, 14].

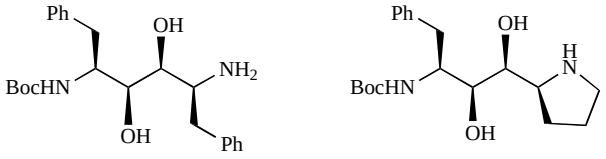



**Fig. (2).** The core isostere.

In this investigation a high-molecular weight poly(ethylene glycol) moiety (PEG, MW = 6000 Da) has been chosen as a linker, even if it has been reported that dimerization inhibitors linked on polyetheric chains are characterized by a reduced biological activity [15]. However this effect should be balanced by the well-known ability of large PEG chains to improve the bioavailability of the conjugated drugs [16].

In literature some conjugates between PEG and some HIV-PR commercial inhibitors nowadays in use (Saquinavir, Indinavir, Nelfinavir) are described [17]; in all these examples PEG renders the conjugate a prodrug, but the release is always very slow independently from the nature of the linkage carrier-drug [18].

On these basis, we decided to focus our attention exclusively on mixed conjugates bearing two different inhibitors on the same polymeric support, with the purpose of overcoming the known reduced activity of the PEGylated drugs with the expected synergic effect due to the presence of two different inhibitors linked on the same biocompatible carrier.

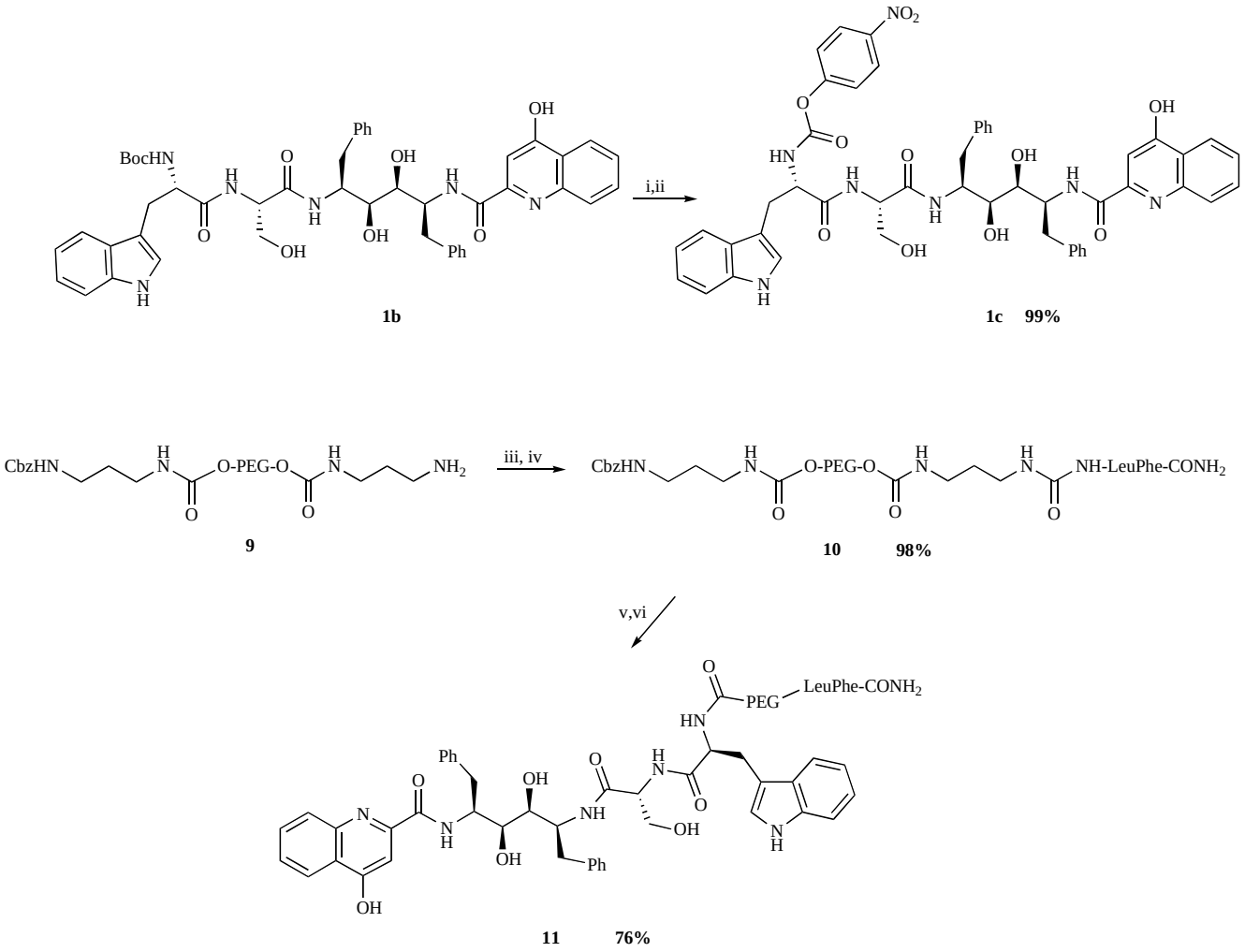
The orthogonally protected diamino PEG **9** was thus synthesized according to a method recently described by Bonora, G.M. *et al*. [19]. This selectively end-modified polymer derivative can be easily modified at its extremities with two different molecules, as demanded for our purposes.

As an example (Scheme **1**), the synthesis of conjugate **11** between dimerization inhibitor **6** and catalytic site inhibitor **1** is described. Dimerization inhibitor **6** is first conjugated to the linker **9** previously activated as NOSu; once deprotected the remaining PEG terminal reacting group, the second inhibitor **1c**, previously activated using *p*$NO_2$-phenyl chloroformate, is conjugated through a similar ureidic linkage. The intermediates and the products were analyzed by $^1$H NMR spectroscopy and their identity and purity evaluated by comparison of signals due to PEG chain and linked molecules; the amount of the free $NH_2$ was also calculated using the colorimetric TNBS test [20]. In all cases, the estimated purity degree of the final compounds was at least 95%.

The same approach was used for the synthesis of **12** and **13**, obtained from the mixed conjugation of **2b** + **5** and of **3b** + **4** (Fig. **3**).

The activity of the three conjugates **11-13** was then evaluated *in vitro* against the HIV-1 PR and compared with the free compounds (Table **1**).

The Phe-Phe isostere and Nelfinavir conjugates **11** and **13** are less active than their parent active site inhibitors, but more active than their dimerization inhibitor precursors. On




**Scheme 1.** i) TFA/DCM, rt; ii) *p*-$NO_2$-Ph chloroformate, DIPEA, $CH_2Cl_2$, rt; iii) *N*,*N*-disuccinimidyl dicarbonate, TEA, $CH_2Cl_2$, rt; iv) **6**, TEA, $CH_2Cl_2$, rt; v) $H_2$, Pd/C, HCl, MeOH, rt; vi) **1c**, TEA, $CH_2Cl_2$, rt

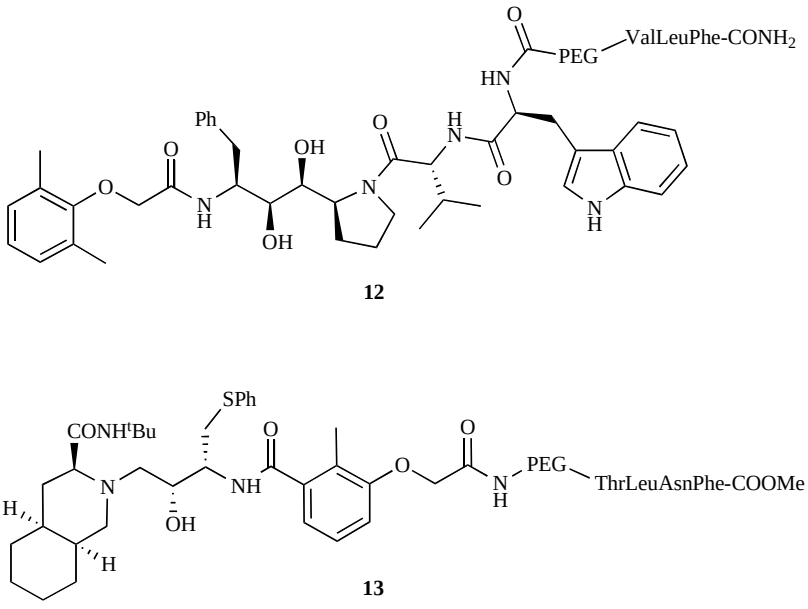



**Fig. (3).** Mixed inhibitors.

the contrary, compound **12** is one order of magnitude more active than its parent Phe-Pro active site inhibitor **2a**, and more than four orders of magnitude than the tripeptide **5**. The conjugate activity is thus heavily dependent upon structure. However, true synergism appears to operate in compound **12**, thus indicating that improved pharmacological properties can be obtained by this approach.

**Table 1. $IC_{50}$ Values of Free and Conjugated Compounds**

| Inhibitor | $IC_{50}$ μM[a] |
|---|---|
| 1a | 4 |
| 2a | 0.0753 |
| 3a | 0.0019 |
| 4 | 660 |
| 5 | 85 |
| 6 | 110 |
| 11 | 35.2 |
| 12 | 0.0062 |
| 13 | 1.2 |

[a]These data were obtained by measuring the initial rates of hydrolysis of the fluorogenic substrate Abz-Thr-Ile-Nle-Phe($NO_2$)-Gln-Arg. Results are the average over at least three independent experiments.

In conclusion, this first data confirm the convenience of the fast and high yielding methodology adopted for the preparation of a new class of mixed HIV inhibitors whose behaviour clearly suggest a likely enhancing of the pharmacological activity through a cooperative effect.

A larger set of derivatives is currently being synthesized in order to gain insight in the structural effects that drive the resulting conjugate activity. At that level extensive pharmacokinetic analysis will be performed as well as the *in vivo* behaviour on MOL T3 type linfoblastoid cells to afford useful data also on their toxicity. Further information will be obtained from the study of their activity in cellular tests, both on *wild type* and mutants HIV viruses, in order to understand and evaluate the PEG effect on the cellular permeability. All these studies will be reported in a following paper focused on the pharmacological features of these new mixed HIV-PR inhibitors.

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[20] The TNBS analysis was made on 2 mg samples of PEG conjugates, accurately weighted and added to a 10 ml flask with 9 ml of borate buffer 0.1 M (pH 9.3) and with 0.25 ml of a 0.03 M solution of TNBS in the same buffer. The volume was adjusted to 10 ml and after 30 min the absorbance at 421 nm ($\varepsilon = 12850$) gave the amount of free $NH_2$ groups.