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Short Communication

Synthesis and biological evaluation of first *N*-alkyl *syn* dimeric 4-aryl-1,4-dihydropyridines as competitive HIV-1 protease inhibitors

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Abstract – A first series of novel *N*-alkyl substituted *syn* dimeric 4-aryl-1,4-dihydropyridines **12**–**17** have been synthesised and evaluated as HIV-1 protease inhibitors in in vitro assays. While the *N*-methyl derivatives **12** and **13** were almost inactive, with IC_{50}-values of about 225 μM, the *N*-benzyl compounds with varied ester groups all exhibited stronger activities, with IC_{50}-values of 11–12 μM for the presently best compounds **16** and **17** with ethyl ester functions. The type of HIV-1 protease inhibition of the novel inhibitors was characterised as competitive. With the increase of observed activity from *N*-methyl derivatives to *N*-benzyl compounds the binding mode may correspond to that of cyclic ureas with hydrophobic interactions of the four aromatic residues to the S1/S1′ and S2/S2′ regions of HIV-1 protease. © 2001 Éditions scientifiques et médicales Elsevier SAS

***Syn* dimeric 4-aryl-1,4-dihydropyridines / HIV-1 protease inhibitor / Competitive inhibition**

1. Introduction

Since the discovery of HIV-1 protease (PR) as a novel target enzyme for the development of HIV-1 protease inhibitors (PI), certain peptidic PIs have been established in HIV-therapy combined with nucleoside analogues or reverse transcriptase inhibitors [1–3]. Resistance development against peptidic PIs, which should be lowered by those combined therapies, is one main problem today because the resistances described earlier against single PIs mutated to certain cross-resistances against all the marketed peptidic PIs [4]. The additional problems of the present peptidic PIs, poor oral bioavailability and severe side effects, just in long-term therapies like cardiovascular morbidity and mortality, strengthened the development of non-peptidic PIs that promised better bioavailability. However, hopeful inhibitors of a first series of cyclic ureas, e.g. **DMP 323** (*figure 1*), failed in clinical trials, with unsatisfactory bioavailabilities [5]. From a series of 4-hydroxy-2-pyrones as non-peptidic PIs with better oral bioavailability, one representative **PNU-140690** (*figure 1*) is presently undergoing clinical trials [6]. Nevertheless, the development of novel non-peptidic lead structures is a great challenge to the drug design of today. Cage dimeric 4-aryl-1,4-dihydropyridines have recently been introduced as a novel class of non-peptidic PIs [7, 8].

N-Substituted cage dimeric 4-aryl-1,4-dihydropyridines **I** possess functional groups similar to those of cyclic ureas (*figure 1*). Certain conformities in the molecular properties of the cage dimeric target structure **I** and cyclic ureas were suggested by molecular modeling [9]. One main functional difference between cyclic ureas and these cage dimers is the urea carbonyl group that directly binds to Asp25A and 25B, replacing a water molecule and the hydroxymethyl groups of the cage dimers, which were also suggested for

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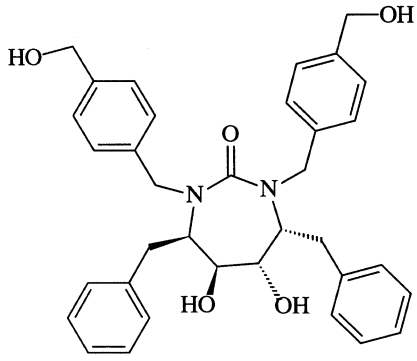
direct binding to those amino acids by molecular modeling.

We now present a first series of *syn* dimeric 4-aryl-1,4-dihydropyridines with target structure **II** supplied with ester carbonyl functions instead of the hydroxymethylene functions. Compared with the cage dimers the ring-opened character of the *syn* dimers provided higher flexibility for the binding of their aromatic residues to the hydrophobic regions S1/S1′ and S2/S2′ of PR. The variously substituted target structure **II** was simply yielded by few reaction steps, thus following a facile synthetic route.

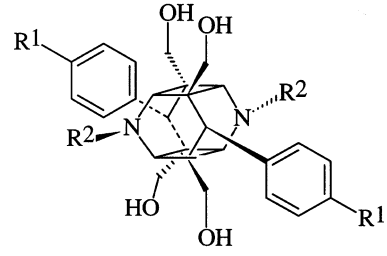
The novel non-peptidic PIs have been evaluated regarding their PI-activity and the type of inhibition of PR in an in vitro system.

2. Chemistry

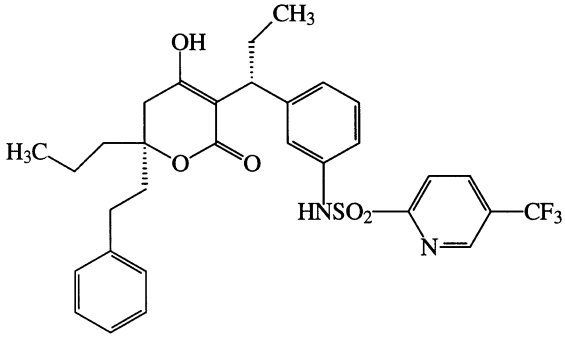
Syn dimer **4** has been given by the solid-state photodimerisation reaction of monomeric 4-phenyl-1,4-dihydropyridine **1** [10] using unfiltered light of Ultra Vitalux lamps® with $\lambda \geq 270$ nm [11] (*figure 2*). Monomeric derivatives **2** and **3**, which were themselves produced by cyclocondensation reactions of benzaldehyde, methyl and ethyl propiolate, respectively, and benzylamine in acetic acid, yielded *syn* dimers **5** and **6** by solution dimerisation reactions besides *anti* dimers **7** and **8** using filtered light at $\lambda > 313$ nm [12] (*figure 2*). The *syn* configuration was proved by the cyclisation reaction of the dimers to cage compounds on irradiation with unfiltered light



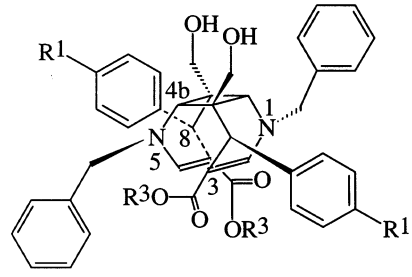
DMP 323



I



PNU-140690



II

Figure 1. Structures of cyclic urea **DMP 323**, 4-hydroxy-2-pyrone **PNU-140690**, cage dimeric 4-aryl-1,4-dihydropyridine **I** and *syn* dimeric target structure **II** (with chosen *N*-benzyl substitution) discussed in the text.

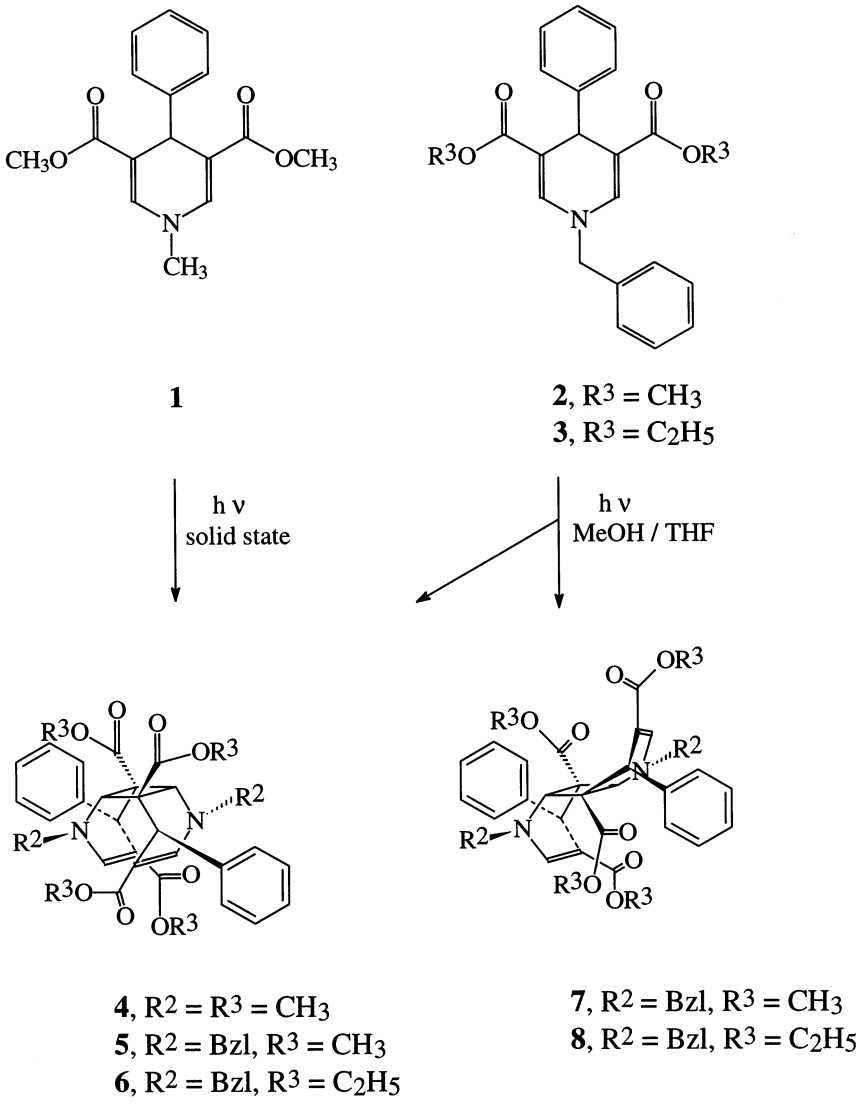


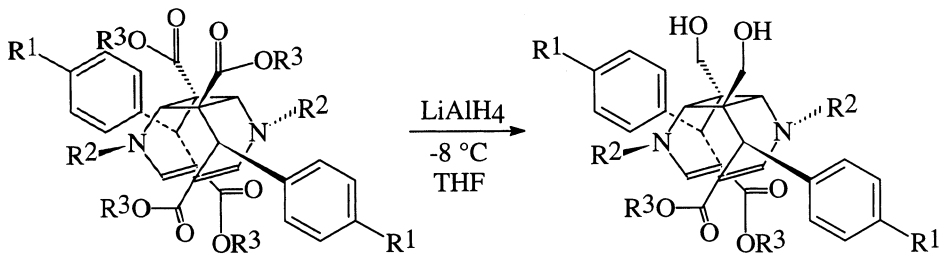
Figure 2. Synthesis of intermediate compounds **4**–**8**.

under excitation of the carbamide ester chromophore as has been demonstrated for reported *syn* dimers **9**–**11**, while *anti* dimers remain stable on further irradiation (A. Hilgeroth, unpublished results).

Selective ester group reductions in all the *syn* dimers **4**–**6** and **9**–**11** to the corresponding alcoholic target structures **12**–**17** succeeded under optimised conditions using lithium aluminium hydride ($LiAlH_4$) at −8°C in dry THF without any affecting of the carbamide ester function (*figure 3*). The vinylogous carbamide ester structure in **12**–**17** was proved spectroscopically by the almost unchanged UV absorption of the carbamide ester chromophore between $\lambda =$ 304–330 nm, the IR absorption bands between $\nu =$ 1669–1679 cm^{-1} as well as by the unchanged chemical shifts of the vinylogous protons at $\delta \sim 7.4$ ppm, compared with the *syn* dimers **4**–**6** and **9**–**11**. (See Section 6 for selected compounds **4** and **12**.)

3. Pharmacology

The in vitro inhibitory activities of the synthesised *syn* dimers **12**–**17** against PR have been evaluated. Saquinavir has been used as the reference compound



4-6, 9-11 **12-17**

	R1	R2	R3	
4	H	CH_3	CH_3	**12**
9	OCH_3	CH_3	CH_3	**13**
5	H	Bzl	CH_3	**14**
10	OCH_3	Bzl	CH_3	**15**
6	H	Bzl	C_2H_5	**16**
11	OCH_3	Bzl	C_2H_5	**17**

Figure 3. Synthesis of target structures **12**–**17**.

with a determined percentage inhibition of 99% at a concentration of 1 μM and a K_i value of 2.1 nM. The IC_{50}-values of the *syn* dimers are presented (*table I*). Due to the poor solubilities of all the derivatives **12**–**17** except **16**, their IC_{50}-values could not be determined directly. They were estimated based on triple inhibitory determinations at 1 (**17**), 6.25 (**14**,**15**) or 50 μM for the other compounds assuming parallel dose–response curves according to Eq. (1). The determination of the presented K_i values (*table I*) followed Ref. [13].

$$\frac{v}{v_{max}} = \frac{IC_{50}}{[I]+IC_{50}} \quad (1)$$

4. Results and discussion

Within the series of investigated methyl ester derivatives **12**–**15** both the *N*-methyl derivatives **12** and **13** were almost inactive, with IC_{50}-values>200 μM. The introduction of *N*-benzyl substituents in **14** and **15** mainly increases the inhibitory activities, with IC_{50}<30 μM and inhibitory constants of about 10 μM. The differences in the activity data from *N*-methyl to *N*-benzyl substitution of the methyl ester derivatives suggest a favourable binding of the aromatic *N*-benzyl substituents to the hydrophobic binding regions S1/S1′or S2/S2′ of PR. Such a binding will not be possible in the case of the small *N*-methyl derivatives. Moreover, for those reported cyclic ureas with hydrophobic binding of all four aromatic residues to PR, *N*-benzyl substituents are also favoured substituents for binding to those hydrophobic regions [5]. The change of ester substitution from methyl to ethyl derivatives in **16** and **17** slightly increases the inhibitory activity, with an IC_{50}-value of 11 μM and K_i of 4.8 μM for the presently best compound **17**. So *syn* dimers **12**–**15** are moderately potent inhibitors of PR.

The novel PIs were proved to be competitive inhibitors of PR. From the Lineweaver–Burk plot of

Table I. HIV-1 protease inhibition of *syn* dimers **12**–**17**.

Compound	IC_{50} (μM)	K_i (μM)
12	254 ± 22.9	110 ± 9.5
13	222 ± 20.5	96 ± 8.8
14	21 ± 1.5	8.7 ± 0.6
15	29 ± 6.3	12.1 ± 2.6
16	12.1 ± 0.9	5.8 ± 0.4
17	11 ± 0.7	4.8 ± 0.3

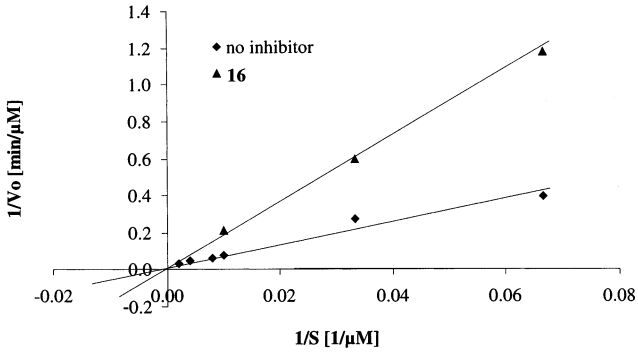


Figure 4. Lineweaver–Burk-Plot for inhibitor **16**. The substrate concentration was varied in the range 15–500 µM. The inhibitor concentration was fixed at 15 µM. From linear fits of the data the parameters V_{max} and $K_{m/app}$ were calculated with V_{max} (µM min^{-1}) = 37±4 and K_m (µM) = 214±38 for the uninhibited reaction and V_{max} (µM min^{-1}) = 45±4 and K_{app} (µM) = 777±98 for the inhibited reaction. The K_i value was determined with 5.7 µM, thus well corresponding to the one given in *table I*.

velocities of substrate cleavage at various substrate concentrations for uninhibited and inhibited reactions with selected *N*-benzyl derivative **16** the maximum velocity of substrate cleavage nearly remains unchanged while the calculated Michaelis–Menten constant increases from 214 to 777 µM (*figure 4*).

5. Conclusions

The results of this study show that the *N*-benzyl substituent as an aromatic one is absolutely necessary for certain binding activities of the novel PIs. The very slight difference in the biological activities of the methyl and ethyl ester derivatives may indicate that there will be no favourable interactions of the varied alcoholic component of the ester groups with the active site of PR. The non-significant protein binding [14] and the poor metabolism of the dimeric, non-peptidic PIs compared with the ureas [15] are advantageous with respect to bioavailability. So the development of this promising class of PIs is strongly encouraged.

6. Experimental protocols

6.1. Chemistry

Commercial reagents were used without further purification. UV spectra were recorded on a Diode Array spectrophotometer 8452A. ^{1}H-NMR (400 or 500 MHz) spectra were measured using tetramethylsilane as internal standard. TLC was performed on E. Merck 5554 silica gel plates. Mass spectra were measured with an AMD 402 mass spectrometer. Elemental analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values and were performed using a Leco CHNS-932 apparatus (*table II*). The synthesis of compounds **9**–**11** was recently reported in Ref. [11] (**9**,**11**) and Ref. [12] (**10**).

Table II. Elemental analyses of compounds.

	C	H	N
2			
Calc.	72.71	5.82	3.85
Found	72.63	5.56	3.73
3			
Calc.	73.64	6.44	3.58
Found	73.27	6.06	3.52
4			
Calc.	66.90	5.92	4.93
Found	66.87	5.82	4.82
5			
Calc.	72.71	5.82	3.85
Found	72.76	5.89	3.99
6			
Calc.	73.64	6.44	3.58
Found	73.34	6.41	3.43
7			
Calc.	72.71	5.82	3.85
Found	72.61	5.67	3.67
8			
Calc.	73.64	6.44	3.58
Found	73.38	6.55	3.32
12			
Calc.	67.16	6.71	5.22
Found	66.95	6.48	4.88
13			
Calc.	65.40	6.69	4.77
Found	65.55	7.00	4.59
14			
Calc.	74.21	6.37	4.12
Found	74.29	6.65	4.10
15			
Calc.	70.57	6.46	3.74
Found	70.71	6.28	3.51
16			
Calc.	75.62	6.63	4.01
Found	75.60	6.64	4.00
17			
Calc.	72.80	6.64	3.69
Found	72.80	6.76	3.67

*6.1.1. Dimethyl 1-benzyl-1,4-dihydro-4-phenylpyridine-3,5-dicarboxylate (**2**)*

Methyl propiolate (1.68 g, 20 mmol), benzaldehyde (1.06 g, 10 mmol) and benzylamine (1.07 g, 10 mmol) were heated in 1 mL of glacial acetic acid on a steam bath for 15 min. The reaction mixture was then poured into ice-water (100 mL), from which 2.5 g (69%) of **2** crystallised on stirring (m.p.: 158–161°C). ^{1}H-NMR ($CDCl_3$): $\delta =$ 3.59 (s, 6H, CH_3OOC), 4.56 (s, 2H, CH_2N), 4.89 (s, 1H, H-4), 7.10–7.42 (m, 12H, aromatic H, H-2, -6). EIMS; m/z: 363 [M^+]. Anal. $C_{22}H_{21}NO_4$ (C, H, N).

*6.1.2. Diethyl 1-benzyl-1,4-dihydro-4-phenylpyridine-3,5-dicarboxylate (**3**)*

Prepared in a way similar to that described for **2** from ethyl propiolate (1.96 g, 20 mmol), benzaldehyde (1.06 g, 10 mmol) and benzylamine (1.07 g, 10 mmol). Yield: 2.8 g (72%). Yellow powder (m.p.: 145–147°C). ^{1}H-NMR ($CDCl_3$): $\delta = 1.17$ (t, 6H, CH_3CH_2 ,$^3J = 7.0$ Hz), 4.08 (q, 4H, CH_3CH_2, $^3J = 7.0$ Hz), 4.46 (s, 2H, CH_2N), 4.91 (s, 1H, H-4), 7.09–7.73 (m, 12H, aromatic H, H-2, -6). ESIMS; m/z: 392 [$M+H^+$]. Anal. $C_{24}H_{25}NO_4$ (C, H, N).

*6.1.3. Tetramethyl 1,5,8,8bβ-tetrahydro-1,5-dimethyl-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,4aβ,7,8aβ(4H,4bβH)tetracarboxylate (**4**)*

Crystalline **1** (1.0 g, 1.74 mmol) with a layer thickness of 1 mm was irradiated as recently described [11]. After three days the reaction product was dissolved in boiling toluene (100 mL), from which 0.71 g (71%) of **4** crystallised on cooling (m.p.: 226–230°C). UV ($CHCl_3$): λ_{max} (log ε) 244 (3.9), 297 (4.35). IR (KBr): ν1732, 1668. ^{1}H-NMR ($CDCl_3$): $\delta = 3.23$ (s, 6H, CH_3N), 3.33 (s, 6H, CH_3OOC–C4a, C8a), 3.55 (s, 6H, CH_3OOC–C3, C7), 3.54 (s, 2H, H-4, -8), 4.38 (s, 2H, H-4b, -8b), 7.00–7.15 (m, 10H, aromatic H), 7.41 (s, 2H, H-2, -6). ESIMS; m/z: 597 [$M+Na^+$]. Anal. $C_{32}H_{34}N_2O_8$ (C, H, N).

*6.1.4. Tetramethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,4aβ,7,8aβ(4H,4bβH)tetracarboxylate (**5**)*

2 (0.40 g, 1.10 mmol) was dissolved in 40 mL methanol–THF under stirring. The solution was irradiated in a quartz flask placed in a copper (II) sulphate bath (1.25 M) with an Ultra-Vitalux® lamp from a distance of 60 cm for about 4 weeks. After reducing the solution volume *syn* dimer **5** crystallised, finally yielding 0.14 g (35%) as white powder (m.p.: 237–240°C). ^{1}H-NMR ($(CD_3)_2SO$): $\delta = 3.02$ (s, 6H, CH_3OOC), 3.43 (s, 6H, CH_3OOC), 3.83 (s, 2H, H-4, -8), 4.19 (s, 2H, H-4b, -8b), 4.49 (d, 2H, CH_BN, $^2J = 15.0$ Hz), 4.90 (d, 2H, CH_AN, $^2J = 15.0$ Hz), 6.86–7.43 (m, 20H, aromatic H), 7.83 (s, 2H, H-2, -6). EIMS; m/z: 726 [M^+]. Anal. $C_{44}H_{42}N_2O_8$ (C, H, N).

*6.1.5. Tetraethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,4aβ,7,8aβ(4H,4bβH)tetracarboxylate (**6**)*

Prepared in a way similar to that described for **5** from **3** (0.40 g, 1.02 mmol). Yield: 0.14 g (36%). White needles (m.p.: 138–145°C). ^{1}H-NMR ($CDCl_3$): $\delta = 0.87$ (t, 6H, CH_3CH_2OOC–C4a, C8a, $^3J = 7.2$ Hz), 1.15 (t, 6H, CH_3CH_2OOC–C3, C7, $^3J = 7.0$ Hz), 3.53 (AMX_3, 2H, CH_3CH_MOOC–C4a, C8a, $^2J = 10.7$ Hz, $^3J = 7.2$ Hz), 3.63 (AMX_3, 2H, CH_3CH_AOOC–C4a, C8a, $^2J = 10.7$ Hz, $^3J = 7.2$ Hz), 3.99 (AMX_3, 2H, CH_3CH_MOOC–C3, C7, $^2J = 10.7$ Hz, $^3J = 7.0$ Hz), 4.05 (s, 2H, H-4, -8), 4.06 (AMX_3, 2H, CH_3CH_AOOC–C3, C7, $^2J = 10.7$ Hz, $^3J =$ 7.0 Hz), 4.46 (s, 2H, H-4b, -8b), 4.59 (d, 2H, CH_BN, $^2J = 14.8$ Hz), 4.71 (d, 2H, CH_AN, $^2J = 14.8$ Hz), 6.88–7.40 (m, 20H, aromatic H), 7.61 (s, 2H, H-2, -6). EIMS; m/z: 782 [M^+]. Anal. $C_{48}H_{50}N_2O_8$ (C, H, N).

*6.1.6. Tetramethyl 1,5-dibenzyl-1,5,8,8bα-tetrahydro-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,4aα,7,8aβ(4H,4bβH)tetracarboxylate (**7**)*

The volume of the mother liquid from the preparation of **5** was further reduced leading to crystallisation of *anti* dimer **7** as a white powder with a total yield of 0.13 g (32%) (m.p.: 251–256°C). ^{1}H-NMR ($CDCl_3$): $\delta = 3.16$ (s, 6H, CH_3OOC–C4a, C8a), 3.52 (s, 6H, CH_3OOC–C3, C7), 4.13 (s, 2H, H-4, -8), 4.38 (d, 2H, CH_BN, $^2J = 15.0$ Hz), 4.46 (s, 2H, H-4, -8), 4.60 (d, 2H, CH_AN, $^2J = 15.0$ Hz), 6.58–7.42 (m, 20H, aromatic H), 7.46 (s, 2H, H-2, -6). EIMS; m/z: 726 [M^+]. Anal. $C_{44}H_{42}N_2O_8$ (C, H, N).

*6.1.7. Tetraethyl 1,5-dibenzyl-1,5,8,8bα-tetrahydro-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,4aα,7,8aβ(4H,4bβH)tetracarboxylate (**8**)*

After separation of **6** the *anti*-dimer **8** crystallises from the solution with a collected yield 0.13 g (32%) as a white powder (m.p.: 231–236°C). ^{1}H-NMR ($CDCl_3$): $\delta = 1.11$ (t, 6H, CH_3CH_2OOC–C4a, C8a, $^3J = 7.2$ Hz), 1.16 (t, 6H, CH_3CH_2OOC–C3, C7, $^3J = 7.2$ Hz), 3.58 (q, 4H, CH_3CH_2OOC–C4a, C8a, $^3J = 7.2$ Hz), 3.94 (AMX_3, 2H, CH_3CH_MOOC–C3, C7, $^2J = 10.7$ Hz, $^3J = 7.2$ Hz), 4.06 (AMX_3, 2H, CH_3CH_AOOC–C3, C7, $^2J = 10.7$ Hz, $^3J =$ 7.2 Hz), 4.17 (s, 2H, H-4, -8), 4.39 (d, 2H, CH_BN, $^2J = 14.7$ Hz), 4.52 (s, 2H, H-4b, -8b), 4.67 (d, 2H, CH_AN, $^2J = 14.7$ Hz), 6.94–7.42 (m, 20H, aromatic H), 7.46 (s, 2H, H-2, -6). EIMS; m/z: 782 [M^+]. Anal. $C_{48}H_{50}N_2O_8$ (C, H, N).

*6.1.8. General procedure for compounds **12–17***

*6.1.8.1. Dimethyl 1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-1,5-dimethyl-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,7(4H, 4bβH)dicarboxylate (**12**)*

4 (0.04g, 0.07 mmol) was dissolved in dry THF (25 mL) and the solution was cooled down to −8°C. Then a solution of lithium aluminium hydride (1.4 mL, 1.4 mmol) in THF (1 M) was added. After 6 h the reaction mixture was hydrolysed with 2 mL of a solution of potassium hydroxide (20%) at 0°C. The water layer was then extracted with 100 mL chloroform three times. The combined extracts were dried over sodium sulphate. On reducing the extraction volume crude **12** crystallised and was recrystallised from methanol–water yielding 0.026 g (71%) of pure **12** as a white powder (m.p.: 255–260°C). UV ($CHCl_3$): λ_{max} (lg ε) 242 (3.4), 330 (3.4). IR (KBr): ν1670. ^{1}H-NMR ($(CD_3)_2SO$): $\delta = 2.94$ ('d', 2H, CH_BOH, $^2J = 10.5$ Hz), 3.11 (s, 6H, CH_3N), 3.15 ('d', 2H, CH_AOH, $^2J = 10.5$ Hz), 3.31 (s, 2H, H-4b, -8b), 3.34 (s, 6H, CH_3OOC), 3.47 (s, 2H, H-4, -8), 4.57 (s, br, 2H, exchangeable, OH), 7.05–7.16 (m, 10H, aromatic H), 7.32 (s, 2H, H-2, -6). ESIMS; m/z: 519 [M+H$^+$]. Anal. $C_{30}H_{34}N_2O_6 \cdot H_2O$ (C, H, N).

*6.1.8.2. Dimethyl 1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-1,5-dimethyl-4,8-bis(4-methoxyphenyl)-cyclobuta[1,2b:3,4b′]dipyridine-3,7(4H,4bβH)-dicarboxylate (**13**)*

Yield: 0.026 g (72%) (m.p.: 210–225°C). ^{1}H-NMR ($CDCl_3$): $\delta = 1.31$ (dd, 2H, exchangeable, OH, $^3J = 8.0$ Hz, 5.0 Hz), 3.20 (s, 6H, CH_3N), 3.21 (s, 2H, H-4b, -8b), 3.28 (dd, after D_2O addition d, 2H, CH_BOH, $^2J = 11.5$, $^3J = 8.0$ Hz), 3.46 (s, 2H, H-4, -8), 3.47 (dd, after D_2O addition d, 2H, CH_AOH, $^2J = 11.5$, $^3J = 5.0$ Hz), 3.51 (s, 6H, CH_3OOC), 3.74 (s, 6H, CH_3O), 6.75–6.90 (m, 8H, aromatic H), 7.39 (s, 2H, H-2, -6). FDMS; m/z: 578 [M$^+$]. Anal. $C_{32}H_{38}N_2O_8 \cdot 0.5H_2O$ (C, H, N).

*6.1.8.3. Dimethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-4,8-diphenylcyclobuta[1,2b:3,4b′]dipyridine-3,7(4H,4bβH)dicarboxylate (**14**)*

Yield: 0.015 g (41%) (m.p.: 222–225°C). ^{1}H-NMR ($(CD_3)_2SO$): $\delta = 3.06$ (dd, after D_2O addition d, 2H, CH_BOH, $^2J = 12.0$, $^3J = 3.1$ Hz), 3.25 (dd, after D_2O addition d, 2H, CH_AOH, $^2J = 12.0$, $^3J = 3.1$ Hz), 3.38 (s, 2H, H-4b, -8b), 3.42 (s, 6H, CH_3OOC), 3.54 (s, 2H, H-4, -8), 4.44 (d, 2H, CH_BN, $^2J = 14.1$ Hz), 4.57 (d, 2H, CH_AN, $^2J = 14.1$ Hz), 4.61 (t, 2H, exchangeable, OH, $^3J = 3.1$ Hz), 7.09–7.43 (m, 20H, aromatic H), 7.49 (s, 2H, H-2, -6). EIMS; m/z: 670 [M$^+$]. Anal. $C_{42}H_{42}N_2O_6 \cdot 0.5H_2O$ (C, H, N).

*6.1.8.4. Dimethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-4,8-bis(4-methoxyphenyl)-cyclobuta[1,2b:3,4b′]dipyridine-3,7(4H,4bβH)-dicarboxylate (**15**)*

Yield: 0.016 g (43%) (m.p.: 235–245°C). ^{1}H-NMR ($CDCl_3$): $\delta = 0.86$ (d, 2H, exchangeable, OH, $^3J = 1.0$ Hz), 3.18 (dd, after D_2O addition, d, 2H, CH_BOH, $^2J = 12.0$, $^3J = 1$ Hz), 3.29 (s, 2H, H-4b, -8b), 3.34 ('d', 2H, CH_AOH, $^2J = 12.0$ Hz), 3.53 (s, 6H, CH_3OOC), 3.60 (s, 2H, H-4, -8), 3.72 (s, 6H, CH_3O), 4.45 (d, 2H, CH_BN, $^2J = 15.0$ Hz), 4.55 (d, 2H, CH_AN, $^2J = 15.0$ Hz), 6.61–6.66 (m, br, 8H, aromatic H of 4-CH_3OPh), 7.26–7.45 (m, 10H, aromatic H of Bzl), 7.54 (s, 2H, H-2, -6). EIMS; m/z: 730 [M$^+$]. Anal. $C_{44}H_{46}N_2O_8 \cdot H_2O$ (C, H, N).

*6.1.8.5. Diethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-4,8-diphenyl-cyclobuta[1,2b:3,4b′]dipyridine-3,7(4H,4bβH)dicarboxylate (**16**)*

Yield: 0.029 g (81%) (m.p.: 261–263°C). ^{1}H-NMR ($(CD_3)_2SO$): $\delta = 1.10$ (t, 6H, CH_3CH_2, $^3J = 7.0$ Hz), 2.91 (dd, after D_2O addition d, 2H, CH_BOH, $^2J = 10.9$, $^3J = 3.9$ Hz), 3.19 (dd, after D_2O addition d, 2H, CH_AOH, $^2J = 10.9$, $^3J = 3.9$ Hz), 3.48 (s, 2H, H-4b, -8b), 3.62 (s, 2H, H-4, -8), 3.82–3.92 (m, 4H, CH_3CH_{AM}, $^2J = 10.9$, $^3J = 7$ Hz), 4.45 (d, 2H, NCH_B, $^2J = 14.3$ Hz), 4.55 (d, 2H, NCH_A, $^2J = 14.3$ Hz), 4.59 (t, 2H, exchangeable, OH, $^3J = 3.9$ Hz), 7.00–7.45 (m, 22H, aromatic H, H-2, -6). ESIMS; m/z: 721 [M+Na$^+$]. Anal. $C_{44}H_{46}N_2O_6$ (C, H, N).

*6.1.8.6. Diethyl 1,5-dibenzyl-1,5,8,8bβ-tetrahydro-4aβ,8aβ-dihydroxymethyl-4,8-bis(4-methoxyphenyl)-cyclobuta[1,2b:3,4b′]dipyridine-3,7(4H,4bβH)-dicarboxylate (**17**)*

Yield: 0.027 g (75%) (m.p.: 257–260°C). ^{1}H-NMR ($(CD_3)_2SO$): $\delta = 1.11$ (t, 6H, CH_3CH_2, $^3J = 7.0$ Hz), 2.92 (dd, after D_2O addition d, 2H, CH_BOH, $^2J = 10.0$, $^3J = 3.1$ Hz), 3.12 (dd, after D_2O addition d, 2H, CH_AOH, $^2J = 10.0$, $^3J = 3.1$ Hz), 3.43 (s, 2H, H-4b, -8b), 3.56 (s, 2H, H-4, -8), 3.66 (s, 6H, CH_3O), 3.81–3.94 (m, 4H, CH_3CH_{AM}, $^2J = 10.7$, $^3J = 7.0$ Hz), 4.43 (d, 2H, NCH_B, $^2J = 14.5$ Hz), 4.61 (d, 2H, NCH_A, $^2J = 14.5$ Hz), 4.61 (t, 2H, exchangeable, OH, $^3J = 3.1$ Hz), 6.54–7.49 (m, 20H, aromatic H). FDMS; m/z: 758 [M$^+$]. Anal. $C_{46}H_{50}N_2O_8$ (C, H, N).

6.2. In vitro assay of PR inhibition

The enzymatic activity of PR was measured by following the cleavage of the substrate H-Lys-Ala-Arg-Val-Leu-*p*-nitrophenylalanine-Glu-Ala-Nle-NH_2 [16]. PR was incubated at 37°C in 0.1 M MES, 0.37 M NaCl and 4 nM EDTA, pH 6.25, with 280 μM substrate in the presence or absence of inhibitors in a photometer cuvette. From the decrease of absorbance at 298 nm, the initial reaction rates were calculated [13]. Compounds were added from stock solutions in DMSO.

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