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Identification of small peptides mimicking the R2 *C*-terminus of *Mycobacterium tuberculosis* ribonucleotide reductase

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Ribonucleotide reductase (RNR) is a viable target for new drugs against the causative agent of tuberculosis, *Mycobacterium tuberculosis*. Previous work has shown that an *N*-acetylated heptapeptide based on the *C*-terminal sequence of the smaller RNR subunit can disrupt the formation of the holoenzyme sufficiently to inhibit its function. Here the synthesis and binding affinity, evaluated by competitive fluorescence polarization, of several truncated and *N*-protected peptides are described. The protected single-amino acid Fmoc-Trp shows binding affinity comparable to the *N*-acetylated heptapeptide, making it an attractive candidate for further development of non-peptidic RNR inhibitors. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Mycobacterium tuberculosis*; ribonucleotide reductase; peptide inhibitors; fluorescence polarization

Introduction

Tuberculosis (TB), caused by the bacteria *Mycobacterium tuberculosis* (*Mtb*), is one of the most common infectious diseases in the world, with an estimated 2 billion people infected. Despite a fall in the incidence rate, TB-related deaths are still on the rise [1]. The current treatment regimen is a course at least 6 months long, leading to poor patient compliance. Added to the fact that multidrug-resistant TB (MDR-TB) is present in virtually all surveyed countries, with an even more resistant form denoted extensively drug-resistant TB (XDR-TB) present in 45 of these, the urgent need for novel drugs and drug targets is made clear [2].

All cellular organisms depend on access to a source of deoxyribonucleotides for synthesis and repair of DNA. This source is renewed by the reduction of ribonucleotides into deoxyribonucleotides, a reaction that can only be carried out *in vivo* by the enzyme ribonucleotide reductase (RNR) [3]. This makes RNR an excellent target, e.g. for inhibiting *de novo* genome synthesis, such as in cancer treatment [4]. In the case of *Mtb*, the essential role of RNR is supported by a transposon mutagenesis study [5]. *Mtb* utilizes a class Ib RNR, where the biologically active unit is composed of a larger R1 homodimer and a smaller R2 homodimer, in an $\alpha_2\beta_2$ complex. R1 and R2 are coded for by the genes *nrdE* (Rv3051c) and *nrdF2* (Rv3048c), respectively [5]. It was shown first by Dawes *et al*. and later confirmed by Mowa *et al*. that these genes are crucial for *Mtb*'s survival, also supporting RNR as a promising antitubercular drug target [6,7].

Besides the two active sites where the reduction of all four species of ribonucleotides takes place, the RNR holoenzyme also contains two specificity sites [8]. These allow the enzyme to be allosterically regulated [9]. In this study, rather than targeting any of the nucleotide binding sites, an alternative approach is used: disrupting the formation of the holoenzyme. Studies have revealed that peptides corresponding to the *C*-terminal tail of the R2 subunit from the *E. coli* [10], mammalian [11], and herpes virus RNR systems [12] can compete for their R2 binding site and thus inhibit RNR activity. It has also been shown that the heptapeptide **1** (Figure 1) derived from the *C*-terminal end of *Mtb* R2 can compete for binding to R1 [13]. With this compound as a starting point, we have reported on the design and synthesis of a set of *N*-acetylated heptapeptide inhibitors of *Mtb* RNR [14]. Unfortunately, our attempts to make shorter inhibitors resulted in a loss of inhibitory potency. The *N*-acetylated pentapeptide retained some inhibition, while the *N*-acetylated tetrapeptide was inactive. Interestingly, structure activity studies on small Fmoc-protected peptides and amino acids have shown that they have significant activity against mammalian RNR [15–17]. To investigate if Fmoc-protected peptides also have good binding properties for *Mtb*, we have now prepared a series of analogues and evaluated them using a competitive fluorescence polarization (FP) assay.

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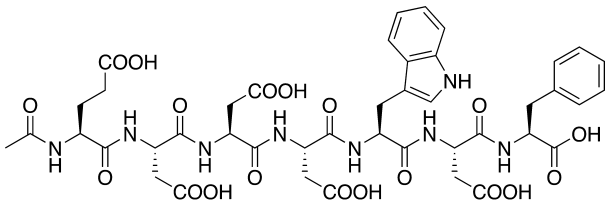

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Figure 1. The *N*-acetylated heptapeptide corresponding to the R2 *C*-terminal end of *Mtb* RNR.

Materials and Methods

General Methods

LC-MS was performed on a Gilson–Finnigan AQA system in ESI mode using a Chromolith SpeedROD RP-18e 4.6 × 50 mm column (Merck) and a CH_3CN/H_2O linear gradient with 0.05% HCOOH. Preparative RP-HPLC was carried out using a Zorbax SB-C8, 5 μm (21.2 × 150 mm) column. The purity of the compounds was determined by analytical RP-HPLC in the following systems (UV detection at 220 nm): (A) ACE 5 C18, 50 × 4.6 mm, H_2O/MeCN gradient with 25 mM NH_4OAc, pH 6.3 and (B) Thermo Hypersil C4, 50 × 4.6 mm, 5 μm, H_2O/MeCN gradient with 0.1% trifluoroacetic acid (TFA). Amino acid analysis was conducted at the Department of Biochemistry, Uppsala University, Sweden. Samples were hydrolyzed with 6 M HCl at 110 °C for 24 h and analyzed with ninhydrin detection. Exact molecular masses were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source.

Peptide Synthesis

Peptides **2**–**6** and **8**–**10** were prepared manually on a solid phase in a disposable syringe equipped with a porous polyethylene filter using standard Fmoc/*t*-Bu-protection. The peptide sequence of the dansylated peptide **17** (Gly-Ser-Gly-Glu-Asp-Asp-Asp-Trp-Asp-Phe) was synthesized with a Symphony instrument (Protein Technologies, Inc., Tucson, AZ, USA) followed by manual dansylation. The starting polymer was H-L-Phe-2-chlorotrityl resin. Removal of the Fmoc group was accomplished by treatment with 20% piperidine in *N*,*N*-dimethylformamide (DMF) for 2 + 10 min. Coupling was performed in DMF, using *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) in the presence of *N*,*N*-diisopropylethylamine (DIEA). Amino acids with the following side chain protection were used: Asp(O*t*-Bu), Glu(O*t*-Bu), Ser(*t*-Bu), Trp(Boc). All peptides prepared by solid phase synthesis were cleaved by treatment of the resin with TFA/H_2O/triethylsilane (95 : 5 : 5) for 1.5 h. The TFA was evaporated and the products were precipitated by addition of diethyl ether. Dipeptides **7** and **16** were prepared in solution as described below. The crude peptides were purified by preparative RP-HPLC and selected fractions were pooled and lyophilized. The peptides were analyzed by HRMS and by two different analytical RP-HPLC systems. The peptide content was determined by amino acid analysis.

General Procedure for Preparation of Peptides 2–6

Peptides **2**–**6** were prepared starting with 500-μmol H-L-Phe-2-chlorotrityl resin. Coupling of the appropriate amino acids (3 equiv) was performed in DMF, using HBTU (3 equiv), in the presence of DIEA (6 equiv), for 3 h. After each coupling step, part of the resin was removed to give the Fmoc-protected peptides of the desired lengths. The crude peptides were purified by preparative RP-HPLC (MeCN/H_2O gradient with 25 mM NH_4OAc, pH 6.3). The overall yields ranged from 25–46%. Analysis data are given in Table 1.

Preparation of Peptide 7

Dipeptide **7** was prepared in solution from the pentafluorophenyl (Pfp) ester derivative, Fmoc-L-Asp(O*t*-Bu)-Pfp on a 100-μmol scale.

Table 1. Analytical data of compounds **2**–**16**

Compound	Amino acid analysis	HRMS [M-H]$^-$ Calcd	HRMS [M-H]$^-$ Found	HPLC purity (%)[a] A	HPLC purity (%)[a] B
2	Asp 3.72, Glu 1.01, Phe 0.99, Trp[b]	1161.3689	1161.3651	99	98
3	Asp 3.81, Phe 1.00, Trp[b]	1032.3263	1032.3256	>99	>99
4	Asp 2.79, Phe 1.00, Trp[b]	917.2994	917.3000	>99	99
5	Asp 1.89 Phe 1.00, Trp[b]	802.2724	802.2730	>99	>99
6	Asp 1.01, Phe 0.99, Trp[b]	687.2455	687.2450	>99	>99
7	Asp 0.99, Phe 1.01	503.1818	503.1815	>99	>99
8	Asp 1.66, Phe 1.00, Trp[b]	786.2775	786.2773	>99	>99
9	Asp 1.61, Phe 1.00, Trp[b]	788.2932	788.2916	>99	>99
10	Asp 1.68, Phe 1.00, Trp[b]	712.2619	712.2634	>99	>99
11	–	386.1392	386.1388	>99	>99
12	–	462.1705	462.1717	98	97
13	–	425.1501	425.1506	>99	>99
14	–	296.0923	296.0918	99	>99
15	–	354.0978	354.0984	>99	>99
16	Asp 2.00	469.1247	469.1235	>99	>99

[a] The purity of the compounds was determined by analytical RP-HPLC in the following systems (UV detection at 220 nm): (A) ACE 5 C18, 50 × 4.6 mm, H_2O/MeCN gradient with 25 mM NH_4OAc, pH 6.3 and (B) Thermo Hypersil C4, 50 × 4.6 mm, 5 μm, H_2O/MeCN gradient with 0.1% TFA.
[b] Not determined.

Coupling with H-L-Phe-O*t*-Bu (1.1 equiv) was performed in DMF, in the presence of DIEA (2.1 equiv), for 30 min. EtOAc was added to the reaction mixture and the organic layer was washed with 1.0 M aqueous HCl, 5% aqueous $NaHCO_3$, dried with $MgSO_4$ and concentrated by rotary evaporation. The residue was treated with TFA/H_2O/triethylsilane (95 : 5 : 1) for 30 min. The crude dipeptide was purified by preparative RP-HPLC (MeCN/H_2O gradient with 25 mM NH_4OAc, pH 6.3). Selected fractions were pooled and lyophilized. The yield was 23%. Analysis data are given in Table 1.

General Procedure for Preparation of Peptides 8–10

Peptides **8**–**10** were generated on a 50-µmol scale. Coupling of the appropriate carboxylic acid derivative (3 equiv), 9-fluoreneacetic acid, 3,3-diphenyl propionic acid or 3-phenyl propionic acid, to H-Asp(O*t*-Bu)-Trp(Boc)-Asp(O*t*-Bu)-Phe-2-chlorotrityl resin, were performed using HBTU (3 equiv) in the presence of DIEA (6 equiv), for 3 h. The crude peptides were purified by preparative RP-HPLC (MeCN/H_2O gradient with 25 mM NH_4OAc, pH 6.3). The total yields ranged from 15 to 25%. Analysis data are given in Table 1.

Fmoc-Protected Amino Acids 11–15

The Fmoc-protected amino acids **11**–**14** were all used as obtained from the suppliers. Fmoc-Asp (**15**) was generated from Fmoc-L-Asp(O*t*-Bu)-OH, by treatment with TFA/H_2O/triethylsilane (95 : 5 : 3) for 30 min, and was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA). All Fmoc-protected amino acids were analyzed by HRMS and by two different analytical RP-HPLC systems. Analysis data are given in Table 1.

Preparation of Peptide 16

Dipeptide **16** was prepared in solution from Fmoc-L-Asp(O*t*-Bu)-Pfp on a 100-µmol scale. Coupling with H-L-Asp(O*t*-Bu)-O*t*-Bu (1.1 equiv) was performed in DMF, in the presence of DIEA (2.1 equiv), for 30 min. EtOAc was added to the reaction mixture and the organic layer was washed with 1.0 M aqueous HCl, 5% aqueous $NaHCO_3$, dried with $MgSO_4$ and concentrated by rotary evaporation. The residue was treated with TFA/H_2O/triethylsilane (95 : 5 : 1) for 30 min. The crude dipeptide was purified by preparative RP-HPLC (MeCN/H_2O gradient with 0.1% TFA). Selected fractions were pooled and lyophilized. The yield was 74%. Analysis data are given in Table 1.

Preparation of Peptide 17

The dansylation of the H-Gly-Ser(*t*-Bu)-Gly-Glu(O*t*-Bu)-Asp(O*t*-Bu)-Asp(O*t*-Bu)-Asp(O*t*-Bu)-Trp(Boc)-Asp(O*t*-Bu)-Phe-2-chlorotrityl resin was performed manually in a disposable syringe on a 75-µmol scale. The polymer was treated with a solution of dansyl chloride (4 equiv) and DIEA (3 equiv) in DMF and was allowed to react for 3.5 h. The dansylated peptide was cleaved and precipitated as described above and was purified by preparative RP-HPLC (MeCN/H_2O gradient with 25 mM NH_4OAc, pH 6.3) to give the dansylated peptide **17** in 17% yield.

Cloning, Expression and Purification of *Mtb* RNR R1

The cloning, expression and purification of the *Mtb* R1 subunit was performed as previously described by Nurbo *et al*. [14]. Prior to concentrating R1, urea and glycerol were added to final concentrations of 1 M and 50%, respectively. The R1 subunit was concentrated to 100 µM, with some batch variation. The protein could then be stored for at least one month at −20 °C without any noticeable loss of activity. Protein concentrations were determined by taking the average of measurements at 280 nm on an Eppendorf Biophotometer and a Thermo Scientific 1000 Nanodrop Spectrophotometer.

Fluorescence Polarization Assay

All lyophilized compounds were solubilized in 100% dimethyl sulfoxide (DMSO) to a stock concentration of 5–25 mM and stored at −20 °C. The assay buffer contained 5 mM HEPES pH 7.0; 0.01% Nonidet P-40 and 100 nM dansylated peptide (**17**). For evaluating the dissociation constant between probe and R1, the protein was serially diluted from a starting concentration of 10 µM down to 0.004 µM. For compound screening, compound concentrations were serially diluted from 100 µM down to 0.01 µM or lower. R1 was added to a final concentration ranging from 0.8 to 1.5 µM. All final assay volumes were 50 µl and set up in Perkin Elmer 384 F OptiPlates. Reactions were set up at room temperature in PCR-strips, mixed by inversion, and allowed to reach equilibrium during 5 min of incubation. Measurements were done on triplicate reactions on a Perkin Elmer Envision 2104 Multilabel Reader using 340 and 535 nm filters for excitation and emission, respectively.

Results and Discussion

In our previous study based on the *N*-acetylated heptapeptide **1**, we monitored the reduction of ribonucleotides to the corresponding deoxyribonucleotides by estimating the reduction of ^{3}H-labeled cytidine 5′-diphosphate (CDP). We have now adopted a more high-throughput binding assay based on FP, which we use in the present study. We have previously shown

Table 2. Binding affinity of compounds **1**–**16**

Compound	Sequence	$K_{D2} \pm$ SD (µM)[a]	r^{2}[b]
1[c]	Ac-Glu-Asp-Asp-Asp-Trp-Asp-Phe	8.3 ± 0.3	0.988
2	Fmoc-Glu-Asp-Asp-Asp-Trp-Asp-Phe	0.7 ± 0.1	0.857
3	Fmoc-Asp-Asp-Asp-Trp-Asp-Phe	2.3 ± 0.1	0.990
4	Fmoc-Asp-Asp-Trp-Asp-Phe	7.6 ± 0.1	0.985
5	Fmoc-Asp-Trp-Asp-Phe	19 ± 3	0.978
6	Fmoc-Trp-Asp-Phe	102 ± 10	0.849
7	Fmoc-Asp-Phe	60 ± 1	0.956
8	9-Fluoreneacetyl-Asp-Trp-Asp-Phe	29 ± 2	0.977
9	3,3-Diphenylpropanoyl-Asp-Trp-Asp-Phe	84 ± 9	0.833
10	3-Phenylpropanoyl-Asp-Trp-Asp-Phe	>150	–
11	Fmoc-Phe	>150	–
12	Fmoc-Dif	51 ± 3	0.932
13	Fmoc-Trp	12.4 ± 1	0.995
14	Fmoc-Gly	>150	–
15	Fmoc-Asp	>150	–
16	Fmoc-Asp-Asp	>150	–

[a] K_{D2} values are the average of three wells with the associated standard deviation. Values >150 µM are estimates only.
[b] The correlation index, used as the parameter to be maximized during the nonlinear curve fit [20–22]. For K_{D2} values >150 mM, no meaningful fit is possible.
[c] Synthesis and characterization reported earlier by Nurbo *et al*. [14].

that at least a hexapeptide was required to achieve micromolar RNR inhibition. In the present study the aim was to investigate if smaller analogues of peptide **1** with the Fmoc protecting group could compete for binding to the *Mtb* R1 subunit. The compounds evaluated are shown in Table 2.

Synthesis of Peptides

The peptides **2**–**6** were generated manually using standard Fmoc/*t*-Bu solid-phase peptide synthesis. The starting polymer was H-Phe-2-chlorotrityl resin, HBTU was used as the activating agent and DIEA was used as the base. The dipeptide **7** was prepared in solution from the activated ester derivative Fmoc-Asp(O*t*-Bu)-Pfp and H-Phe-O*t*-Bu. Peptides **8**–**10** were generated from partially protected H-Asp-Trp-Asp-Phe-2-chlorotrityl resin by coupling with the appropriate carboxylic acid derivative. After completion of the syntheses, a mixture of TFA, water and triethylsilane were used to cleave the target peptides from the resin. The crude products were precipitated in diethyl ether and purification by RP-HPLC gave homogeneous products. The Fmoc-protected amino acids **11**–**14** were all obtained from commercial suppliers and were used without further purification. Fmoc-Asp (**15**) was produced from Fmoc-Asp(O*t*-Bu)-OH by treatment with TFA and the dipeptide **16** was prepared in solution from Fmoc-Asp(O*t*-Bu)-Pfp and H-Asp(O*t*-Bu)-O*t*-Bu. All target compounds were analyzed by HRMS and gave ions consistent with their molecular masses. According to analyses by two different HPLC systems, the target compounds were at least 97% pure. The fluorescent peptide used for displacement measurements in the FP assay, Dansyl-Gly-Ser-Gly-Glu-Asp-Asp-Asp-Trp-Asp-Phe (**17**), was synthesized as described for the other peptides synthesized on solid phase, followed by removal of the terminal Fmoc protecting group and derivatization with dansyl chloride in the presence of DIEA.

Competitive Fluorescence Polarization Assay

The affinity of compounds **1**–**16** as binders to the *Mtb* R1 subunit was evaluated by use of a competitive FP assay. The method has the advantage of acting on systems in equilibrium, so there is no need for time measurements to determine initial velocities. Furthermore, since it is a method for studying binding behavior, no catalysis has to take place. The use of fluorescence polarization for screening small-molecule inhibitors of protein–protein interactions is exhaustively detailed in two

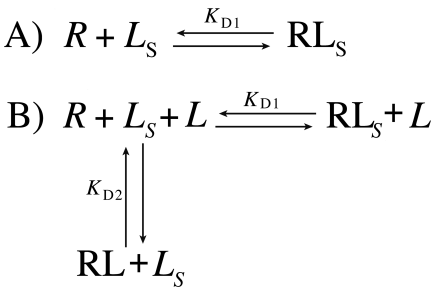

Figure 2. (a) Direct binding model. (b) Complete competitive model. The following definitions are used: *R*, free R1; *L*, free unlabeled ligand (the tested compound); L_S, free labeled ligand (the probe, i.e. the dansylated heptapeptide); K_{D1}, dissociation constant of the interaction between free R1 and the probe; K_{D2}, dissociation constant of the interaction between free R1 and unlabeled ligand. This definition is identical to that of K_i which is used elsewhere in the literature.

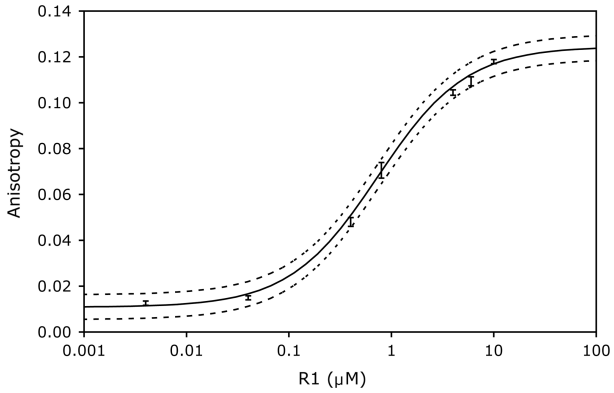

Figure 3. A plot of the direct binding of 100 nM dansylated heptapeptide to increasing concentration of R1. Fitting the data to Equations (6) and (39) from Roehrl *et al*. [19] yielded the solid curve with the following parameters: $Q = 3.1$; $A_B = 0.124 \pm 0.0004$; $A_F = 0.011 \pm 0.001$; $K_{D1} = 2.2 \pm 0.02$ μM. The dotted curves enclose a 95% confidence interval of the fit.

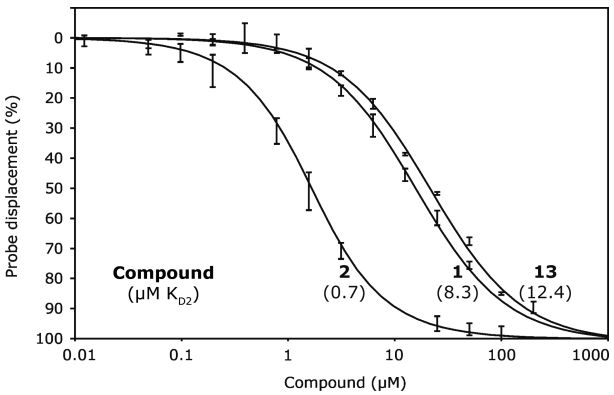

Figure 4. Displacement of dansylated heptapeptide by compounds **1**,**2** and **13** according to the complete competitive model.

back-to-back publications by Roehrl *et al*. [18,19]. As a first step, the direct binding model (Figure 2(a)) was used to calculate a dissociation constant for the interaction between the dansylated heptapeptide (**17**) and R1. The K_{D1} was evaluated to be 2.2 ± 0.02 μM. Figure 3 illustrates the sigmoidal curve produced by increasing concentrations of R1 while keeping the concentration of probe constant. With K_{D1} determined, it is possible to determine K_{D2} values for each compound, i.e. the dissociation constant of the interaction between a compound and R1. This is done by applying the complete competitive model, as shown in Figure 2(b). K_{D2} values for the evaluated compounds are presented in Table 2. Dissociation curves for compounds **1**, **2** and **13** are shown in Figure 4.

Truncation of Fmoc-Protected Peptides

To investigate the effect of replacing the *N*-acetyl group with Fmoc, a series of *N*-terminally truncated peptide analogues were synthesized and evaluated (Table 2). The *N*-acetylated heptapeptide **1** was first reanalyzed using the FP assay and the K_{D2} value evaluated to be 8.3 ± 0.3 μM. The corresponding Fmoc-protected peptide **2** showed a 10-fold improvement in binding affinity with $K_{D2} = 0.7 \pm 0.1$ μM. Encouraged by this result, we prepared and evaluated the truncated Fmoc-protected peptides **3**–**7**. Although each deleted amino acid resulted in a 3–5-fold

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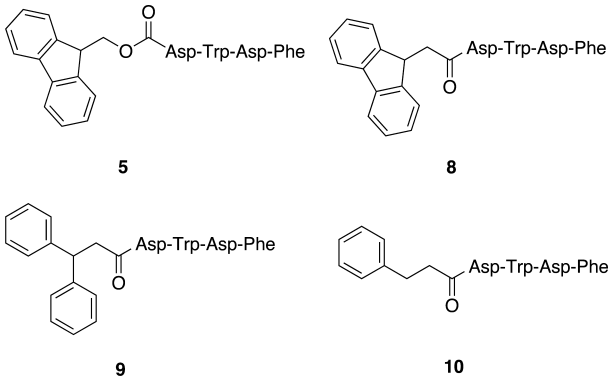

Figure 5. *N*-terminal groups investigated in this study.

loss in affinity, the dipeptide **7** ($K_{D2} = 60 \pm 1$ μM) had surprisingly good binding affinity. Thus, small Fmoc peptides were capable of competing for binding to R1.

Effect of Introducing Different *N*-terminal Groups

Next, we were interested to see if this positive binding effect was associated only with the Fmoc group. Thus, we decided to investigate other *N*-terminal groups. The tetrapeptide (**5**) was chosen as the model compound. Three tetrapeptides were acylated with different *N*-terminal groups to give peptides **8**–**10** (Figure 5). Peptide **8**, acylated with 9-fluoreneacetic acid, turned out to have only slightly less affinity than the corresponding Fmoc-protected tetrapeptide **5** ($K_{D2} = 19 \pm 3$ μM), with a K_{D2} value of 29 ± 2 μM. Making the *N*-terminal ring system less rigid as in peptide **9** ($K_{D2} = 84 \pm 9$ μM) gave a drop in affinity, whereas introduction of a smaller ring system (**10,** $K_D > 150$ μM) resulted in a more dramatic loss of binding affinity. These results showed that the Fmoc group could be substituted for other similar groups with almost retained affinity.

Fmoc-Protected Amino Acids

Furthermore, inspired by the retained affinity of dipeptide **7**, we decided to investigate single Fmoc-protected amino acids. Interestingly, truncation down to single Fmoc-Phe (**11**) was not tolerated. However, the use of amino acids with larger side chains as in Fmoc-protected 3,3-diphenylalanine (**12**) and Fmoc-Trp (**13**) was more successful, with K_{D2} values of 51 ± 3 μM and 12.4 ± 1 μM, respectively. Fmoc-Gly (**14**) and Fmoc-Asp (**15**) which are smaller and less hydrophobic were not able to compete for binding to R1. The Fmoc-protected dipeptide **16** was also prepared to investigate if additional charged residues might be favorable for binding. However, the affinity was too low for a dissociation constant to be determined. Surprisingly, Fmoc-Trp (**13**) has a binding affinity comparable with the acetylated heptapeptide **1** and is therefore an interesting compound for further discovery of small inhibitors targeting *Mtb* RNR.

Conclusions

An assay suitable for screening potential *Mtb* RNR inhibitors has been developed. Furthermore, we have demonstrated that the introduction of an *N*-terminal Fmoc group in the heptapeptide, corresponding to the *C*-terminus of *Mtb* R2 gives an increase in binding affinity. This effect was also seen for shorter peptides and truncation down to single Fmoc-protected amino acids was tolerated. The identification of Fmoc-Trp as a potential lead compound, with binding affinity comparable to the acetylated heptapeptide, is especially promising. This small molecule represents an excellent starting point for the development of non-peptidic inhibitors of *Mtb* RNR.

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