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Pitfalls in the synthesis of fluorescent methotrexate oligopeptide conjugates

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Abstract Methotrexate (MTX) conjugates with poly[Lys(DL-Ala$_m$)] based polymeric polypeptides are efficient against Leishmania donovani parasite infection, but the mechanism of the effect is not known yet. We prepared therefore the 5(6)-carboxyfluorescein (Cf) labelled oligopeptide [Cf-K(AaAa)][†] and the correspoding MTX-conjugates [Cf-K(MTX-AaAa)] as model compounds for structure-activity experiments. The conjugate aimed to be synthesized with solid-phase methodology on MBHA resin with Boc strategy, using Fmoc-Lys(Boc)-OH. However, various side-reactions were identified. Here we report three problems observed during the synthesis as well as solutions developed by us:

a) Unexpected cyclopeptide-formation with the lactone-carboxylic group of the Cf was detected, when Cf was attached to the α-amino group of the Lys residue on solid phase. This was avoided by changing the order of Cf incorporation with using Fmoc/Dde strategy. Alternatively, we have built the peptide with Fmoc strategy on solid phase first and performed the labelling with Cf-OSu subsequently in solution.

b) During HF cleavage of the protected conjugates, MTX was demonstrated to form adducts with anisole and *p*-cresol scavengers, and the TMSOTf cleavage methodology was also found to be inadequate due to the large number of side products formed. We report here that using Fmoc/Dde strategy is an appropriate method to circumvent the cleavage with HF or TMSOTf.

c) During the coupling of MTX with oligopeptide, structural and stereo isomers are formed. We have described here the suitable conditions of HPLC separation of these products.

[†] Abbreviations: a: *D*-alanine, A: *L*-alanine

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Keywords
oligopeptide conjugates, methotrexate, labelling with carboxyfluorescein, Fmoc/Dde strategy

Abbreviations

Boc	Tert-butyloxycarbonyl protecting gorup
BSA	Bovine serum albumin
Cf	5(6)-Carboxyfluorescein
Cf-OSu	5(6)-Carboxyfluorescein N-hydroxy succinimide ester
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
Dde	1-(4,4-Dimethyl-2,6-dioxacyclohexylidene) ethyl protecting group
DEE	Diethyl ether
DIC	N,N′-Diisopropylcarbodiimide
DMF	Dimethylformamide
EDT	1,2-Ethanedithiol
Fmoc	9-Fluorenylmethoxycarbonyl protecting group
HF	Hydrogen fluoride
HOBt	1-Hydroxybenzotriazole
MBHA	4-Methylbenzhydrylamine
MTX	Methotrexate
TFA	Trifluoroacetic acid
TMSOTf	Trimethylsilyl trifluoromethanesulfonate

Introduction

Leishmaniasis is one of the most critical tropical diseases, as recognized by the World Health Organisation (WHO Report, 2000). The treatment has not been fully resolved yet. Chemotherapy is available; but the efficiency is threatened by the growing incidence of resistant parasites, together with the frequent and severe side-effects [Sundar, S. et al., 2007] [Das, M. et al., 2013].

A promising method for destroying the parasites in the cells could be the inhibition of the pterine or folate metabolism that is different from human metabolism via selective inhibition of the pteridine reductase, thymidylate synthase or dihydrofolate reductase enzymes [Schüttlekopf, A.W. et al., 2005]. Methotrexate (MTX, ((2S)-2-[[4-[(2,4-diaminopteridin-6yl)methylmethylamino]benzoyl]amino]pentanedioic acid, **Fig. 1**) could be considered as one of the specific inhibitors of dihydrofolate reductase enzyme. It was proven that MTX conjugated to bovine serum albumin or maleyl-BSA was internalised by macrophages and killed effectively the intracellular parasites [Mukhopadhyay, A. et al., 1990].

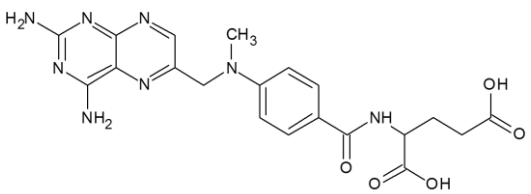


Fig. 1 Methotrexate

Conjugates prepared in our laboratory with efficient anti-parasitic effect containing carrier molecules are based on poly[Lys(DL-Ala$_m$)], (signed as AK‡) polymeric polypeptide. It contains a poly[L-Lys] backbone (the average degree of polymerization = 60-200) and an oligo[DL-Ala] side chain (on average 3-4 Ala/Lys) per monomeric unit. More, effective derivatives of this family of polypeptides with different amino acids in the side chain exhibiting different chemical properties (poly[Lys(X$_i$-DL-Ala$_m$)], XAK‡ or poly[Lys(DL-Ala$_m$-X$_i$)], AXK‡) were described [Hudecz, F. et al., 1993].

‡Nomenclature of branched chain polypeptides was used in accordance with the recommended nomenclature of graft polymers. Short codes of the polypeptides are based on one letter abbreviation of amino acids. X is a required amino acid in the side chain; m represents the average number of amino acids in the side chain; i represents average degree of substitution of amino acid X (i≤1) [IUPAC-IUB, 1984].

Our aim was to synthesize the appropriate compounds corresponding to the monomeric unit of AK and its Cf-labelled MTX conjugates. Namely, Cf-K(AaAa)-NH_2 and Cf-K(MTX-AaAa)-NH_2 peptide were aimed to perpare for structure-activity studies (e.g. chemical stability, enzymatic degradation, antiparasitic activity).

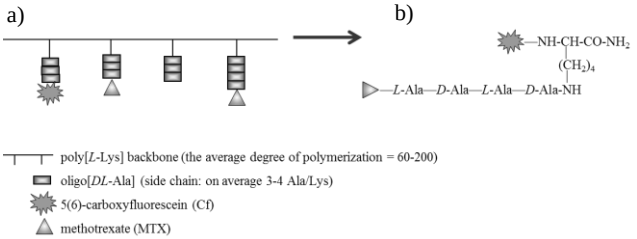


Fig. 2 The schematic structure of Cf-AK-MTX polymer polypeptide conjugate (a) and of the peptide conjugate (b) corresponding to a monomeric unit

Here we report on three pitfalls identified during the synthesis of the model peptides and conjugates , as well as solutions developed by us.

Methods

General: The structures of the peptides were verified with electrospray ionization mass spectrometry (ESI-MS) and UV spectrophotometry. The peptides were purified by Reversed Phase High-performance Liquid Chromatography (RP-HPLC) (97% <). ESI-MS spectra were recorded on Bruker Daltonics Esquire 3000+ ion trap mass spectrometer. The reactions were monitored by RP-HPLC (Phenomenex Synergi C12, 4 μm, 120 Å, 4,6×300 mm column using eluent A: 0.1% TFA/H_2O, and eluent B: 0.1% TFA/80% acetonitrile-H_2O (V/V), and gradient elution 20-80% B (5-20 min) and UV detection: λ=220 nm). The conjugates were purified by RP-HPLC on Phenomenex Jupiter C18, 5μm, 10×300 mm column. Gradient elution 5-100% B (5-60 min) for the crude product and 10-100% B (5-55 min) for the isolated isomer and UV detection (λ=220nm) were applied.

Results and discussion

The Cf-K(MTX-AaAa)-NH_2 oligopeptide conjugate as a model compound (**Fig. 2**) was synthesized on solid phase with conventional peptide synthesis strategy. First, we have chosen Boc/MBHA strategy, using Fmoc-Lys(Boc)-OH as the first coupled amino acid. The side chain of the conjugate was built on the ε-amino group of the protected lysine residue with Boc-Ala followed by unprotected MTX. For coupling DIC/HOBt assisted activation was used. For the removal of the Boc group 33% TFA/DCM (V/V), while for the deprotection of the N^{α}-Lys 30% piperidine/ 70% DMF (V/V) was utilized. First, the Fmoc-Lys(Boc)-OH was coupled to the resin, then the Fmoc group was cleaved and Cf was attached to the N^{α}-amino group of the lysine. It was followed by incorporating the alanine side residues also protected by Boc group and then the MTX. Finally, the conjugate was cleaved from the resin with HF using scavengers (0,5 g *p*-cresol or anisole/10 ml HF).

During the synthesis we observed serious side-reactions, outlined below together with the interpretation and the

solution.

a) Acylation with the carboxyl group of Cf

Unexpected deletion sequences were observed during the attachment of Boc-protected alanine residues if Cf was present at the α-amino-group of the resin attached lysine as demonstrated by MS spectra derived from the crude product (**Fig. 4**).

The likely explanation of the presence of deletion sequences could be the tautomeric carboxylic form of the fluorescein moiety (**Fig. 3**) and its reaction with free amino group, which resulted in the formation of cyclopeptide intermediates.

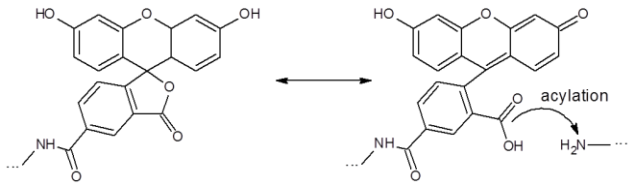
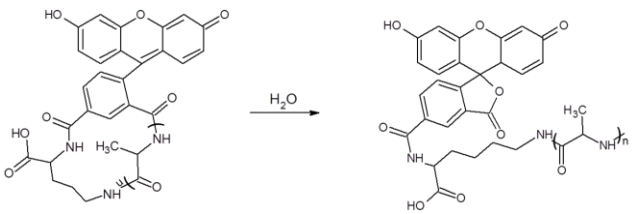
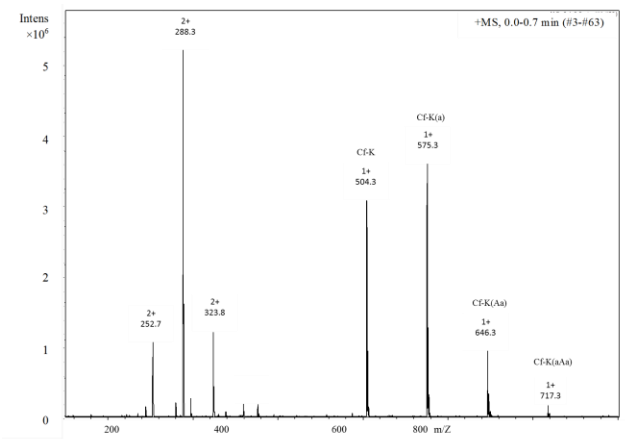


Fig. 3 Tautomers of 5(6)-carboxyfluorescein

These cyclic derivatives prevent the incorporation of the incoming amino acid and hydrolysed during the HF cleavage, thus short peptides with deletion sequences are present in the final products (**Fig. 4**).



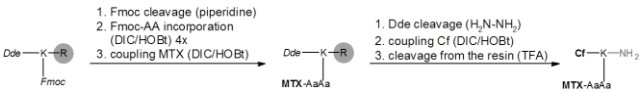
a)



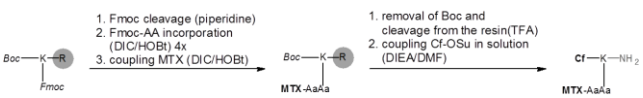
b)

Fig. 4 Hydrolysis of the cyclic peptides (a) and their ESI-MS spectrum (b)

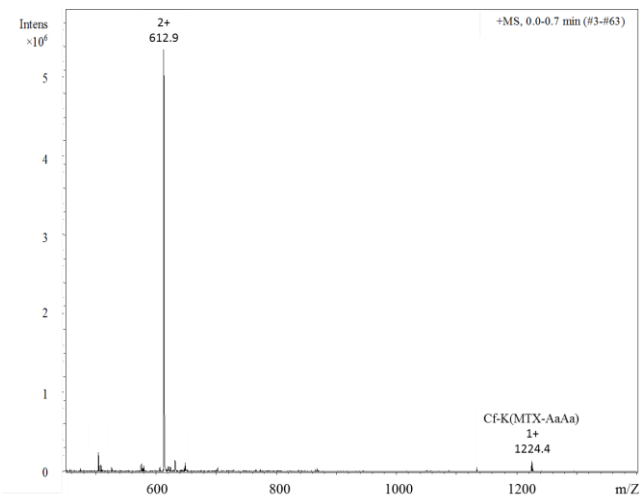
In the state-of-the-art conjugation practice the lacton bound carboxylic group of Cf is expected to be inactive towards amide bond formation. By the synthesis of this peptide we demonstrate, that under some conditions the reactivity of this carboxylic group cannot be neglected. We propose two strategies to avoid the formation of these cyclopeptides. First, by changing the order of Cf incorporation. Instead of the attachment Cf to the Lys(Boc)-Resin, its coupling could be performed as the last step of the synthesis after the removal of the Fmoc protection of the assembled peptide conjugate (Fmoc-K(MTX-AaAa)-Resin). For this we have successfully used the Fmoc/Dde strategy (**Scheme 1**). Another alternative solution is the solution-phase labelling of the free K(MTX-AaAa)-NH_2 peptide using Cf-OSu reagent (**Scheme 2**). By using either method we completed the synthesis of the aimed product with >97% purity (**Fig. 5**) with acceptable yields (>28%).



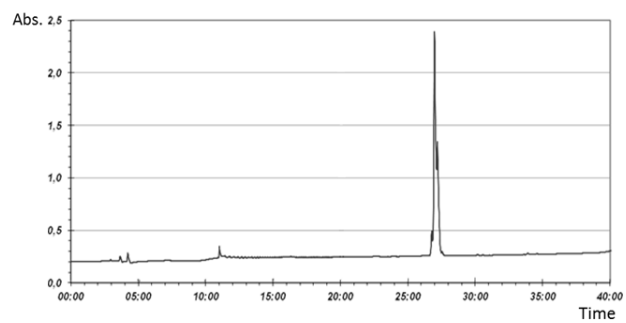
Scheme 1 Changing the order of Cf incorporation



Scheme 2 Labelling with Cf-OSu in solution



a)



b)

Fig. 5 ESI-MS spectrum (a) and RP-HPLC chromatogram (b) of the product

b) Failure of Boc strategy for the synthesis

For labelling the conjugate with Cf-OSu in solution (**Scheme 2**), first we synthesized the K(MTX-AaAa) peptide derivative on MBHA resin with Boc strategy. During cleavage from the resin with liquid HF we used different scavengers. We observed adduct formations with the scavenger *p*-cresol as well as with anisole as evidenced by ESI-MS spectrum (**Fig. 6**).

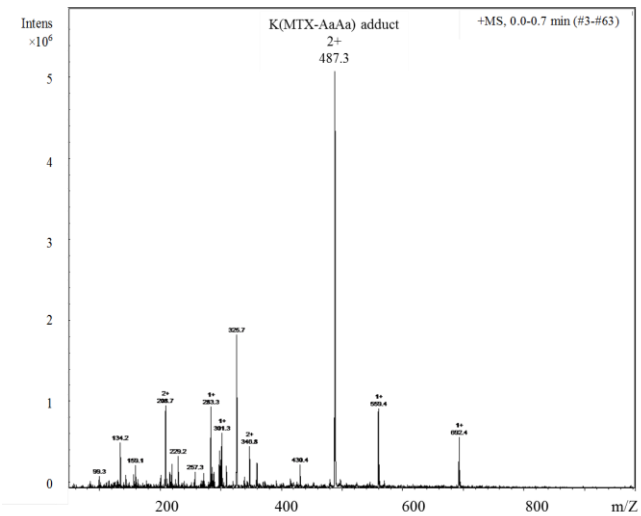


Fig. 6 ESI-MS spectrum from the *p*-cresol adduct with K(MTX-AaAa) M_{av} =973,7

Therefore it was concluded that Boc strategy with HF cleavage seems to be inadequate for the synthesis of MTX containing peptide conjugates, because the sensibility of the MTX aromatic moiety on carbocation attack.

Based on the ESI-MS spectrum of the isolated product we have postulated the structure one of the possible adducts (**Fig. 7**). Further studies are needed to clarify the exact position of substitution.

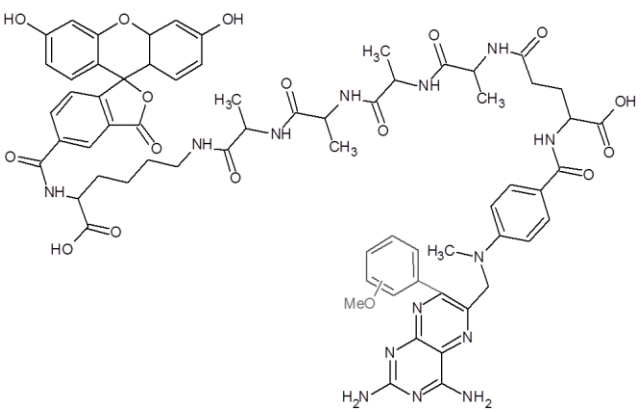


Fig. 7 A hypothesized side-product with anisole

In order to avoid the adduct formation we have tried TMSOTf based cleavage without success. We observed the formation of a complex mixture with more than 10 components and the target compound was not identifiable with RP-HPLC analysis (data not shown).

In order to avoid the harsh cleavage conditions that are not compatible with MTX we utilised Fmoc/Dde strategy on MBHA resin with Rink-amide spacer (**Scheme 1**). The mild cleavage circumstances (95% TFA/ 2,5% water/ 2,5% TIS (V/V/V)) does not cause side-reactions, thus the aimed peptide was possible to be synthesized with high purity (>97%) and yield (>28%).

c) Formation of MTX isomers

The RP-HPLC analysis of MTX-containing conjugates (MTX-AaAa, K(MTX-AaAa), Cf-K(MTX-AaAa)) showed three peaks of approx. equal intensity implying of isomer formation around the glutamic acid moiety of MTX.

The formation of such MTX isomers were studied earlier [V Subr et al., 1997]. The presence of the α and γ MTX isomers in approximately equal quantities is not surprising, thus the α and γ carboxylic groups of MTX have about the same reactivity under the used DIC/HOBt acylation system (**Fig. 8**). Furthermore, the lack of an urethane type protecting group at the α amino group of the MTX could result in racemisation around the alpha carbon, when coupling is done on the α carboxyl.

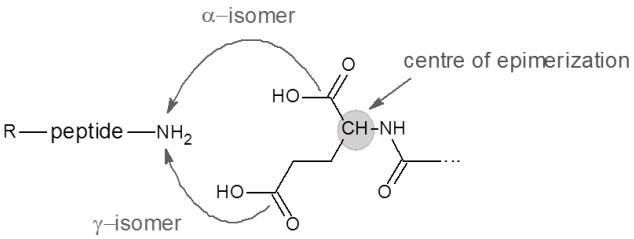


Fig. 8 Formation sites of MTX-peptide isomers

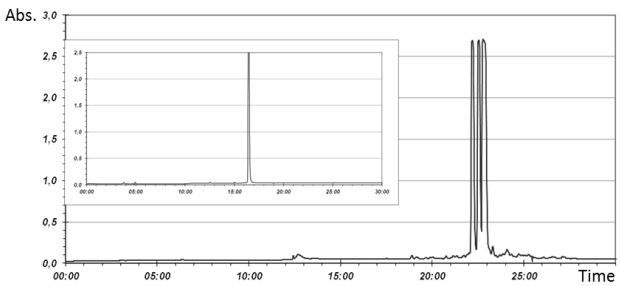


Fig. 9 RP-HPLC chromatogram of the crude MTX-AaAa peptide conjugate product (gradient elution 5-100% B (5-60 min) and UV detection (λ=220nm) were applied) and one of the purified isomers (insert, gradient elution 10-100% B (5-55 min).

We observed that changing the coupling conditions does not alter the isomeric composition significantly, thus the most practical way to obtain the isomerically pure MTX-peptide conjugate was to separate the isomers by HPLC. All three MTX peptide isomers (α L/D and γ L) isolated after semi-preparative RP-HPLC separation (**Fig. 9**) were characterized by ESI-MS. Co-injection experiments

together with molecular mass values support the presence of all isomers.

Discussion

In our study presented here we identified the following possible side reactions that may interfere with the successful synthesis of MTX-oligopeptide conjugates:

a) the generally inert lacton-bound carboxylic group of Cf is proved to be responsible for acylation side-reactions in the presence of DIC/HOBt activation. In orer to circumvent this problem the coupling of Cf should be the last step of conjugate synthesis.
b) MTX is proved to be sensitive for HF/TMSOTf cleavage conditions, thus these conditions should be avoided.
c) the formation of three MTX isomers in about equal amounts are demonstrated. The isomerically pure model peptides obtained by HPLC can be useful to clarify the significance of this side reaction in the pharmacological behavior of the product. The formation of such isomer conjugates are known from previous studies [A Nagy et al., 1993] in the case of peptide hormones.

The results presented here can be utilized predominantly to study comparatively the biological properties (e.g. the effect on cell proliferation, cytotoxicity) of the peptides and the peptide-conjugates (**Table 1**). We wish to understand the fate of the active MTX-branched polypeptide conjugates reported earlier [Gy Kóczán et al., 2002] in the cell. Therefore we aim to utilize the MTX-oligopeptide conjugates as monomeric unit and follow its uptake as well as intracellular traffic and degradation. These experiments will be accomplished by e.g. fluorescent microscopy examinations, flow-cytometry measurements and cell proliferation test Furtherrmore, the observations described could help to improve the synthesis of MTX-polymeric polypeptide conjugates with fluorophore labels.

Table 1 The synthesis strategy used and the chemical characteristics of peptides and conjugates

Compounds	Strategy			R_t	M_{av}
	Boc	Fmoc	Dde/ Fmoc	(min)	calc/meas ESI $[M+H]^+$
AaAa-NH_2	n.t.	+	n.t.	10.4	301.33/302.3
MTX-AaAa-NH_2	-	+	n.t.	22.1/22.2/22.3	737.77/738.3
K(aAaA)-NH_2	+	+	n.t.	11.5	429.50/430.4
K(MTX-aAaA)-NH_2	-	+	n.t.	24.6/25.0/25.3	865.97/866.5
Cf-K(aAaA)-NH_2	+	+	n.t.	25.4	787.82/788.6
Cf-K(MTX-aAaA)-NH_2	-	-	+	27.2/27.3/27.5	1224.26/1224.4

+: successful synthesis
-: unsuccessful synthesis
n.t.: not tested

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study protocol was approved by the Review Board of the UMCG (METc 2008/186) and was in adherence to the Declaration of Helsinki.

References

Clegg, J.A.; Hudecz, F.; Mező, G.; Pimm, M.V.; Szekerke, M.; Baldwin, R.W. (1990) Carrier design: biodistribution of branched polypeptides with a poly (L-lysine) backbone. *Bioconjugate Chemistry* 1: 425-430.

Das, M.; Saudagar, P.; Sundar, S.; Dubey, V.K. (2013) Miltefosine-unresponsive Leishmania donovani has a greater ability than miltefosine-responsive L. donovani toresist reactive oxygen species. *FEBS Journal* 280: 4807-4815.

http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish/en/

Hudecz, F.; Clegg, J.A.; Kajtar, J.; Embleton, M.J.; Pimm, M.V.; Szekerke, M.; Baldwin R.W. (1993) Influence of carrier on biodistribution and in vitro cytotoxicity of methotrexate-branched polypeptide conjugates. *Bioconjugate Chemistry* 4: 25-33.

Kóczán, Gy.; Ghose, A.C.; Mookerjee, A.; Hudecz, F. (2002) Methotrexate conjugate with branched polypeptide influences Leishmania donovani infection in vitro and in experimental animals. *Bioconjugate Chemistry* 13: 518-524.

Mukhopadhyay, A.; Basu, S.K. (1990) Enhanced intracellular delivery of methotrexate by a receptor mediated process. *Biotechnol Appl Biochem.*12: 529-36.

Nagy, A.; Szőke, B.; Schally, A.V. (1993) Selective coupling of methotrexate to peptide hormone carriers through a y-carboxamide linkage of its glutamic acid moiety: Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate activation in salt coupling. *Proc. Natl. Acad. Sci. USA* 90: 6373-6376.

Schüttlekopf, A.W.; Hardy, L.W.; Beverley, S.M.; Hunter, W.N. (2005) Structures of Leishmania major Pteridine Reductase Complexes Reveal the Active Site Features Important for Ligand Binding and to Guide Inhibitor Design. *J. Mol. Biol.* 352: 105–116.

Sundar, S.; Olliaro, PL. (2007) Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. *The Clin. Risk Manag.* 3: 733–740.

General protocol of Boc strategy: For the synthesis MBHA resin was used (capacity 1,12 mmol/g). The resin was swelled in DCM for 3×10 minutes and treated with 33% TFA/DCM (V/V) solution for 2+20 minutes, then washed with DCM 5×, as neutralization with 10% DIEA/DCM (V/V) solution 4× and again with DCM 4×. After that the peptide was built on the resin. For the amino acid and MTX coupling DIC/HOBt coupling agents were used (amino acid : DIC : HOBt = 3,5eq : 3eq : 3eq) in DMF. The reaction time of coupling was 60 minutes. After each coupling a wash with DMF 6× and DCM 6× was done. For controlling the success of the coupling ninhydrin-test was used. Based on this method all couplings were successful (>99.5% conversion). Cleaving of the Boc group was performed by 33% TFA/DCM (V/V) solution for 2+20 minutes. After the cleavage the resin was washed with DCM 5×, as neutralization with 10% DIEA/DCM (V/V) solution 4× and again with DCM 4×. The Fmoc protecting group was removed with 2% piperidine + 2% DBU/DMF (V/V) solution for 2+2+5+10 minutes, then a wash with DMF 6× and then with DCM 6× was applied. Before cleavage the peptide from the resin it was washed with MeOH and DEE, and the resin with the peptide was dried during a night in desiccator.

Cleavage the peptide from the resin with HF was done in a suitable teflon equipment for 120 minutes, in the presence of p-cresol or anisole scavengers (0,5 g scavenger/10 ml HF was used). Then the peptide was precipitated by 50 ml cooled DEE, filtered, and washed 3× with DEE on the filter. The peptide was dissolved in 10% acetic acid solution, separated from the resin with filtration. The solution was lyophilised to isolate the product.

For the TMSOTf cleavage a mixture of TMSOTf/tioanisole/EDT/ p-cresol/TFA (1/0,5/0,25/0,25/3 (V/V/V/V/V)) solution was used for 120 minutes at RT. After the cleavge 50-60 ml cooled DEE was added and the mixture was centrifuged. The peptide was dissolved in 10% acetic acid solution and it was lyophilised to isolate the product.

The general protocol for Fmoc strategy: For the synthesis MBHA resin with Rink-Amide linker was used (capacity 0,56 mmol/g). The resin was swelled in DCM for 2×10 minutes. Then the Fmoc protecting group of the resin was cleaved by 2% piperidine + 2% DBU/DMF (V/V) solution for 2+2+5+10 minutes, then the resin was washed with DMF 6× and DCM 6×. After that the peptide was built on the resin. For the amino acid and MTX coupling DIC/HOBt coupling agents were used (amino acid : DIC : HOBt = 3,5eq : 3eq : 3eq) in DMF. The reaction time of coupling was 60 minutes. After each coupling a wash with DMF 6× and DCM 6× was done. For controlling the success of the coupling ninhydrin-test was used. The cleavage of the Fmoc protecting group of the amino acids was done with the same method as in the case of the cleavage of the resin's Fmoc group. Before cleavage the peptide from the resin it was washed with MeOH and DEE, and the resin with the peptide was dried during a night in desiccator.

For the cleavage the peptide from the resin TFA/H_2O/TIS solution (0,95/0,025/0,025 (V/V/V)) was used for 120 minutes at RT. Then the mixture was filtered into 50-60 ml cooled DEE. The solution mixture was removed by evaporation. The peptide was dissolved in 10% acetic acid solution and lyophilised to isolate the product.

Synthesis of Cf-K(MTX-AaAa)-NH_2 peptide conjugate:

In solution: The K(MTX-aAaA)-NH_2 peptide conjugate was synthesized on Rink-Amide MBHA resin with Fmoc strategy. For the synthesis Boc-Lys(Fmoc)-OH, Fmoc-D-Ala-OH, Fmoc-L-Ala-OH and MTX was used. First the Boc-Lys(Fmoc) was coupled to the resin, than the side chain was built on the ε amino group of the lysine. The remove of Boc group and the cleavage from the resin was done by TFA in the presence of H_2O and TIS. Then the purified K(MTX-AaAa)-NH_2 peptide conjugate was dissolved in DMF and Cf-OSu was coupled in the presence of DIEA basis (peptide : Cf-OSu : DIEA = 1eq : 1,5eq : 1eq). The peptide was purified by RP-HPLC.

On solid phase: The synthesis was done on Rink-Amide MBHA resin with Fmoc/Dde strategy. For the synthesis Dde-Lys(Fmoc)-OH, Fmoc-D-Ala-OH, Fmoc-L-Ala-OH, MTX and Cf was used. First the Dde-Lys(Fmoc)-OH was coupled to the resin, then the side chain was built on the ε amino group of the lysine, and finally the Dde group was cleavaged and the Cf was coupled. The Dde protecting group was removed by 2% H_2N-NH_2.H_2O/DMF (V/V) solution for 6×5 minutes, then wash with DMF 6× and DCM 6× was applied. The peptide was purified by RP-HPLC.