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# Synthesis of oligotuftsin-based branched oligopeptide conjugates for chemotactic drug targeting

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**Abstract:** The synthesis and chemotactic properties of a new class of branched oligopeptide-based conjugates are described. Tetratuftsin derivatives containing chemotactic formyl tripeptides (For-MLF, For-NleLF or For-MMM) in branches were prepared by stepwise solid-phase peptide synthesis. The influence of the composition and ionic charge of the carrier-branched oligopeptide on the chemotactic behaviour of the conjugate was studied in *Tetrahymena pyriformis*. Conjugates with methotrexate (Mtx) as a drug component was also prepared. For this, a GFLGC spacer, cleavable by cathepsin B, was used. The spacer with *N*-terminal methotrexate was coupled to the chloroacetylated chemotactic carrier molecule by thioether bond formation. The chemotactic activity and cytotoxity of Mtx conjugates were also studied. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** drug targeting; chemotactic peptide; tetratuftsin; methotrexate; conjugation; thioether linkage; chemotaxis; cytotoxicity

## INTRODUCTION

The development of target-specific delivery systems for various drugs is still a challenge. Natural and synthetic macromolecules as well as cell-penetrating oligopeptides have been investigated as carriers of bioactive compounds and of reporter molecules for intracellular transport. Both types of carriers can be classified according to the presence or absence of a recognition motif [1]. In contrast to macromolecules, oligopeptides with moderate molecular mass (<10 kDa) usually cannot enter cells by fluid phase or adsorptive endocytosis. Therefore, oligo- or polypeptides without a recognition unit are frequently conjugated with a targeting moiety (e.g. peptide hormones). This type of carrier, recognized by specific receptors, can be taken up by receptor-mediated mechanisms.

In this study, we focus on the development of conjugates for chemotactic drug targeting (CDT). In these constructs, chemotactic peptides function as targeting moieties. Chemotactic responsiveness is a general character of a wide variety of motile subpopulations of cells belonging to different tissues/organs of vertebrates. The novel approach, CDT, deals with the selective chemoattractant ability of the chemoattractant-carrier-drug conjugates on the target cells and provides a new 'physiologically' relevant and targeted way of drug delivery. For example, *N*-formylmethionyl bacterial metabolite peptides are known to constitute a well-characterized class of chemoattractants for neutrophils [2,3]. The presence of stereospecific receptors of these peptides on the surface of the target cells has also been demonstrated [4]. It was proved that the formyl group is crucial for retention of high bioactivity, but the esterification of the *C*-terminal carboxylic group is permitted; no loss of activity for rabbit neutrophils has been observed [5,6]. One of the most active compounds of this group is the peptide For-Met-Leu-Phe-OH. The replacement of Met by Nle, which is a favoured change in peptide analogue design to avoid the oxidation side reaction of Met, did not cause any significant change of the chemotactic activity of the peptide [7,8]. In this study, three *N*-formyl peptides (For-Met-Leu-Phe, For-Nle-Leu-Phe and For-Met-Met-Met tripeptide) were utilized as chemotactic recognition moieties in the carrier structure.

As carrier we applied a sequential oligopeptide on the basis of an immunologically active peptide derived from canine tuftsin (TKPK) and elongated with Gly. The oligotuftsin used in our study consists of four repeat units of the tuftsin derivative $[TKPKG]_4$ (T20), which is non-toxic, non-immunogenic and exhibits tuftsin-like effects (e.g. immunostimulatory and chemotactic activity) [9]. A similar oligotuftsin carrier was used for preparation of conjugates of herpes simplex virus epitope peptides [10], antitumoricidal GnRH-III [11] and $\beta$-amyloid epitope peptides [11,12]. Chemotactic *N*-formyl peptides were attached to the $\varepsilon$-amino group of lysine residues in position 4 of all four units. The construct containing the For-Met-Leu-Phe tripeptide

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was further modified by formylation or succinylation at the $\varepsilon$-amino group of lysine residue at position 2. Thus carriers with cationic, neutral or anionic charge properties were obtained.

Methotrexate as a drug was used as the third component of the conjugate in our experiments. Methotrexate (Mtx, L-amethopterin hydrate, L-4-amino-$N^{10}$-methylpteroyl-glutamic acid), a folic acid antagonist, has high therapeutic value in various clinical conditions. Since its introduction in 1948 [13] for the treatment of acute lymphoblastic leukaemia and osteogenic sarcoma, it has become widely used in cancer chemotherapy [14] and also in rheumatoid disorders [15,16]. Its inhibitory potential against a group of intracellular parasites (Leishmania) of macrophages was also proved [17,18]. Mtx was also one of the first bioactive compounds covalently attached to natural and synthetic macromolecules [1]. For the present studies, Mtx was connected to the carrier with chemotactic moiety through a GFLG spacer sequence that is cleavable by lysosomal enzymes (e.g. cathepsin B) [19].

In this paper, we report the syntheses and characterization of a novel class of bioconjugates composed of tetratuftsin and chemotactic peptides with or without methotrexate. The chemotactic properties as well as the cytotoxicity of the conjugates were studied in *Tetrahymena pyriformis GL*. This eukaryotic ciliated protozoan is frequently used for studying chemotaxis and also for signalling hormone–receptor interactions. These cells carry receptors and secret hormones homologous to the organisms at a higher rank of evolution [20,21].

## MATERIALS AND METHODS

Amino acid derivatives were purchased from Reanal (Budapest, Hungary) or NovaBiochem (Läufelfingen, Switzerland), while MBHA and Rink Amide MBHA resins were purchased from NovaBiochem. Scavengers, coupling agents and cleavage reagents (*p*-cresol, *m*-cresol, *p*-thiocresol, thioanisole, phenol, ethanedithiol (EDT), dithiothreitol (DTT), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*′-diisopropylcarbodiimide (DIC), diisopropyl-ethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), piperidine, trifluoroacetic acid (TFA) and hydrogen fluoride (HF) were Fluka (Buchs, Switzerland) products. Acetic anhydride, dimethylsulfoxide (DMSO) and solvents for synthesis were obtained from Reanal. Acetonitrile for purification, similar to MTT, was ordered from Sigma-Aldrich Kft. (Budapest, Hungary). Methotrexate was from Lederle Laboratories (Gosport, UK). Formic acid trichlorophenyl ester (For-OTcp) and chloroacetic acid pentachlorophenyl ester (ClAc-OPcp) were prepared in our laboratory from the appropriate acid and phenol (Fluka products) with DCC according to the method described by Martinez *et al.* [22]. The reference chemotactic formylated tripeptides (For-MLF, For-NleLF, and For-MMM) were prepared earlier in our laboratories by SPPS using standard Fmoc chemistry.

### Synthesis of Tetratuftsin Conjugates: Ac-(TKPK-(For-X)G)$_4$-NH$_2$(X=MLF (1), NleLF (2), MMM (3))

The tetratuftsin oligopeptide was built up on an MBHA resin (0.62 mmol/g capacity) using standard Boc chemistry as described earlier [11]. The following side chain–protected amino acids were used: Thr(Bzl), Lys[Z(2Cl)] in position 2, and Lys(Fmoc) in position 4 of the repeat units. After completion of the synthesis of the carrier backbone, the Fmoc group was removed selectively and the branches were built up on every second Lys residues. The chemotactic peptide derivative (For-MLF, For-NleLF and For-MMM) was introduced step-by-step using Fmoc strategy. For effective coupling, 12 equiv of Fmoc-amino acid derivatives calculated to the resin capacity were applied. The synthetic protocol was as follows: (i) DMF washing of the resin ($3 \times 0.5$ min); (ii) removal of Fmoc group with 2% DBU-2% piperidine in DMF ($2 + 2 + 5 + 10$ min); (iii) DMF washing ($8 \times 0.5$ min); (iv) coupling of 12 equiv Fmoc-amino acid derivative – DIC – HOBt (60 min); (v) DMF washing ($3 \times 0.5$ min); (vi) DCM washing ($2 \times 0.5$ min) and (vii) ninhydrin assay. After the removal of the last Fmoc group from the *N*-terminal of the branches, the formyl group was attached to the free $\alpha$-amino groups using 20 equiv trichlorophenyl formiate (For-OTcp) in DMF for 3 h. The peptide was cleaved from the resin simultaneously with the removal of the remaining protecting groups with liquid HF in the presence of *p*-cresol and DTT (10 ml HF – 1g *p*-cresol – 0.1 g DTT) at 0 °C for 90 min. The crude products were purified by Reverse Phase High-performance Liquid Chromatography (RP-HPLC) and the pure compounds were characterized by analytical HPLC, electrospray ionization mass spectrometry (ESI-MS) (Table 1) and amino acid analysis (data are not shown).

### Synthesis of Tetratuftsin Conjugates Ac-(TK(X)PK-(For-MLF)G)$_4$-NH$_2$(X=For (4) or Succ(5))

Formylation of the free $\varepsilon$-amino groups of compound (**1**) was carried out in DMF solution (10 mg/ml peptide

**Table 1** Characteristics of peptides and conjugates

| Code | HPLC[a] $R_t$ (min) | ESI-MS[b] calculated [M] | ESI-MS[b] found [M] |
|---|---|---|---|
| **1** | 26.0 | 3783.7 | 3783.4 |
| **2** | 27.2 | 3711.5 | 3711.3 |
| **3** | 22.2 | 3792.0 | 3791.8 |
| **4** | 32.5 | 3895.8 | 3895.5 |
| **5** | 34.1 | 4184.1 | 4183.6 |
| **6** | 33.4 | 3948.9 | 3948.6 |
| **7** | 24.5 | 930.1 | 930.4 |
| **8** | 25.2 | 930.1 | 930.4 |
| **9** | 25.5 | 930.1 | 930.4 |
| **10** | 32.6 | 5738.4 | 5739.2 |
| **11** | 32.4 | 5738.4 | 5737.8 |

[a] RP-HPLC column: Phenomenex Synergy $C_{12}$ (4 μm, 80 Å, $250 \times 4.6$ mm); eluents: 0.1% TFA/water (A), 0.1% TFA/acetonitrile–water 80:20, v/v (B); flow rate: 1 ml/min; gradient: 0 min 0% B, 5 min 0% B, 50 min 90% B.
[b] ESI-MS was performed on Bruker Daltonics Esquire 3000 Plus mass spectrometer.

concentration) using 8 equiv For-OTcp. The reaction mixture was stirred for 24 h and the reaction was followed by HPLC. After completeness, DMF was evaporated and the product (Ac-[TK(For)PK(For-MLF)G]$_4$-NH$_2$) (**4**) was purified by RP-HPLC.

For succinylation, Ac-[TKPK(For-MLF)G]$_4$-NH$_2$ was dissolved in 0.2 M $NH_4HCO_3$ solution at pH 6.5 (10 mg/ml peptide concentration) and 12 equiv succinic anhydride was added. The reaction proceeded overnight and was monitored by HPLC. The product (Ac-[TK(Succ)PK(For-MLF)G]$_4$-NH$_2$) (**5**) was purified directly by RP-HPLC. Compounds **4** and **5** were characterized by ESI-MS and HPLC (Table 1.).

## Synthesis of Branched Oligopeptides with *N*-Terminal Lys in the Backbone; ClAc-K(ClAc)-(TKPK(For-NleLF)G)$_4$-NH$_2$(6)

First Boc-Lys(Boc)-OH was attached to the *N*-terminus of H-[T(Bzl)K[Z(2Cl)]PK(Fmoc)G]$_4$-MBHA using DCC/HOBt activation. Prior to the removal of Boc-groups from both $\alpha$- and $\varepsilon$-amino groups of the *N*-terminal Lys, the NleLF sequence was introduced onto the side chains of the appropriate Lys residues using Fmoc chemistry, as described above. After completion of the synthesis of the chemotactic branches, the Boc-groups from *N*-terminal Lys were removed with 33% TFA in DCM. After the washing and neutralization (10% DIEA in DCM) of the resin, chloroacetylation was performed using 10 equiv of chloroacetic acid pentachlorophenyl ester (ClAc-OPcp) in DMF. The coupling was performed two times for 2 h at RT. The chloroacetylated peptide was removed from the resin with liquid HF using *m*-cresol and *p*-thiocresol scavenger mixture (HF – *m*-cresol – *p*-thiocresol = 10 ml : 0.5 ml : 0.5 g) as suggested for haloacetylated peptide derivatives [23]. The crude product was purified by RP-HPLC as described below, and the pure compound was characterized by analytical HPLC and ESI-MS (Table 1).

## Synthesis of Mtx-GFLGC-NH$_2$

The Mtx-containing GFLGC peptide was synthesised on Rink Amide MBHA resin (0.67 mmol/g capacity) using Fmoc chemistry as described above. The trityl group was used for the side chain protection of Cys. Methotrexate was attached to the peptide resin with BOP and HOBt reagents (2 equiv each) in the presence of 4 equiv DIEA. The peptide was cleaved from the resin with TFA-phenol-thioanisole-EDT-water (10 ml : 0.75 g : 0.5 ml : 0.25 ml : 0.5 ml) mixture at RT for 1.5 h. The resin was filtered out and the crude product was precipitated with ice-cold dry diethyl ether. The precipitate was separated by centrifugation and washed three times with diethyl ether. The solid material was dissolved in acetic acid followed by freeze-drying. Three main peaks were observed in the HPLC chromatogram corresponding to compounds in which the methotrexate was present through the $\gamma$- (**7**) or $\alpha$-carboxylic (**8**) group. The third peak (compound **9**) proved to be the D-isomer of compound **8**, in which instead of L-, D-glutamic acid was present in the methotrexate moiety. According to the AUC (area under curve), the ratio of the peptide derivatives **7** : **8** : **9** was 56.6 : 32.9 : 10.5. After separation and lyophilisation, peptides were isolated by lyophilisation and characterized by analytical HPLC and ESI-MS (Table 1).

## Conjugation of ClAc-K(ClAc)-(TKPK(For-NleLF)G)$_4$-NH$_2$with Mtx-GFLGC-NH$_2$(7 or 8)

Peptide ClAc-K(ClAc)-[TKPK(For-NleLF)G]$_4$-NH$_2$ (**6**) was dissolved in 0.1 M Tris buffer (pH 8.2) at 1 mg/ml peptide concentration, and Mtx-GFLGC-NH$_2$ was added to the solution in solid form at regular intervals for 3 h. The conjugation reaction was monitored by analytical HPLC. When the reaction was completed, the reaction mixture was acidified to pH 2 and purified directly by RP-HPLC. Conjugation of ClAc-K(ClAc)-[TKPK(For-NleLF)G]$_4$-NH$_2$ with $^{\gamma}$Mtx-GFLGC-NH$_2$ or $^{\alpha}$Mtx-GFLGC-NH$_2$ resulted in compounds **10** and **11** respectively. The characteristics of pure compounds are provided in Table 1.

## Reverse Phase High-performance Liquid Chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) HPLC system using a Phenomenex Synergy $C_{12}$ column (250 × 4.6 mm I.D.) with 4-µm silica (80 Å pore size) (Torrance, CA) as stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80 : 20, V/V)) was used at a flow rate of 1 ml/min at ambient temperature. Peaks were detected at $\lambda = 220$ nm.

The crude products were purified on a semipreparative Phenomenex Jupiter $C_{18}$ column (250 × 10 mm I.D.) with 10-µm silica (300 Å pore size) (Torrance, CA). Flow rate was 4 ml/min. The same eluents as described above with an appropriate linear gradient were applied.

## Amino Acid Analysis

The amino acid composition of peptides and conjugates was determined by amino acid analysis using a Beckman Model 6300 analyser (Fullerton, CA, USA). Prior to analysis samples were hydrolysed in 6 M HCl in $N_2$ atmosphere at 110 °C for 24 h.

## Electrospray Ionization Mass Spectrometry (ESI-MS)

The identification of the products was achieved by mass spectrometry. ESI-MS was performed with a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) mass spectrometer, operating in continuous sample injection at 4 µl/min flow rate. Samples were dissolved in 50% acetonitrile–water mixture. Mass spectra were recorded in positive mode in the $m/z$ 200–1500 range.

## Cells and Culturing

*T. pyriformis GL* cells, maintained in 0.1% yeast extract containing 1% Bacto tryptone (Difco, Michigan, USA) medium at 28 °C, were used in the logarithmic phase of growth. Density of samples was $10^4$ cell/ml.

## Assay of Chemotaxis

The chemotactic ability of *Tetrahymena* cells was evaluated using a two-chamber capillary chemotaxis assay [24], which

was modified accordingly [25]. Tips of an eight-channel micropipette filled with the test substances served as the inner chamber of the system. The outer chamber consisted of a microtitration plate filled with the model cells. The incubation time was 20 min. This relatively short time facilitated the measuring of pure gradient, directed chemotactic responses and prevented the contamination of the samples from randomly running chemokinetic responder cells [26]. The concentration dependence of the chemotactic response was determined in the $10^{-12}$–$10^{-6}$ M range. In concurrent runs, a pure PPY (proteose peptone yeast) medium served as control substance. The control samples were evaluated in parallel in each case to eliminate the undesirable disturbances elicited by spontaneous mutations. After incubation the samples were fixed in 4% formaldehyde containing PBS. The number of cells was determined using a Neubauer haemocytometer. Each data point represents the average of 10 parallel experiments.

### Cytotoxicity

*Tetrahymena* cultures were treated with Mtx-containing conjugates **10** and **11** as well as their components Mtx, $^{\gamma}$Mtx-GFLGC (**7**) and Ac-[TKPK(For-NleLF)G]$_4$-$NH_2$ (**2**). Cells were treated in the concentration range of $10^{-10}$–$10^{-6}$ M for 48 h. Control groups were maintained in a PPY medium only. Cells at density of $10^3$ cells/ml were placed into wells of 96-well microtitration plates containing the test substance in sterile conditions. After 48 h MTT assay was applied. Formazan crystals were solubilized in DMSO, and the colorimetric measurement was carried out by an ELISA reader (Labsystems Multiscan MS-Reader, Helsinki, Finland) at $\lambda = 540$ and $\lambda = 620$ nm. Each data point represents the average of eight parallel measurements.

### Statistical Evaluation of Data

Data shown in the figures represent the averages and ±SD values. The statistical analysis was done by the application of ANOVA of Origin 4.0.

## RESULTS AND DISCUSSION

In this work, we report on the preparation of a novel class of branched chemotactic oligopeptides with the aim of selecting carrier molecules for chemotactic drug targeting. In the model conjugate designed (Figure 1), oligotuftsin modified by *N*-formyl tripeptides (For-MLF, For-NleLF or For-MMM) was used as chemotactic carrier to attract target cells. Conjugation of the selected molecule with Mtx produced compounds with preserved cytotoxicity and chemoattractant properties.

### Synthesis

The backbone of the carrier was built up from repeated units of a tuftsin derivative having chemotactic activity itself [9]. For the synthesis of the carrier conjugates with *N*-formyl peptides, Boc chemistry and orthogonal protecting groups (Z(2Cl) and Fmoc) for the side chain of Lys residues were used as outlined in Scheme 1. After selective removal of Fmoc protection, the *N*-formyl peptides (For-MLF (**1**), For-NleLF (**2**) or For-MMM (**3**)) were introduced as branches on the side chain of deprotected Lys residues using Fmoc strategy. The branched peptides were cleaved from the resin

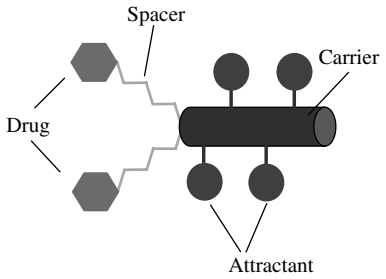
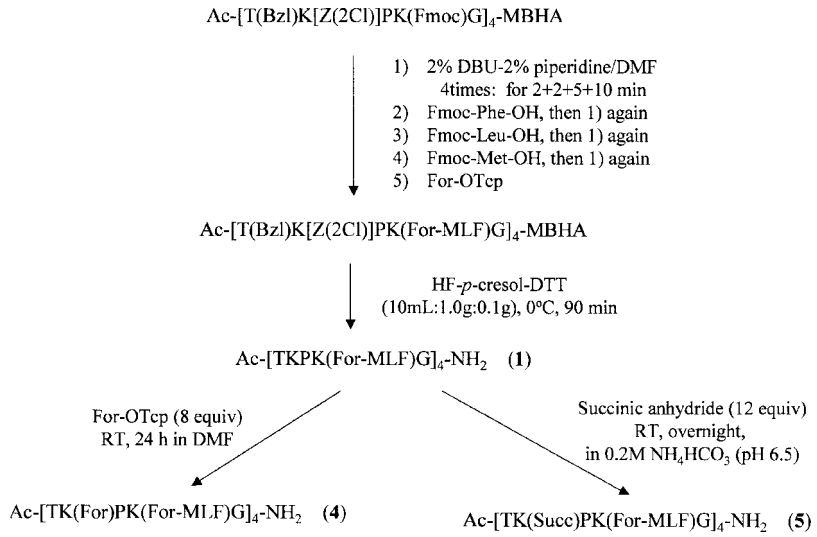


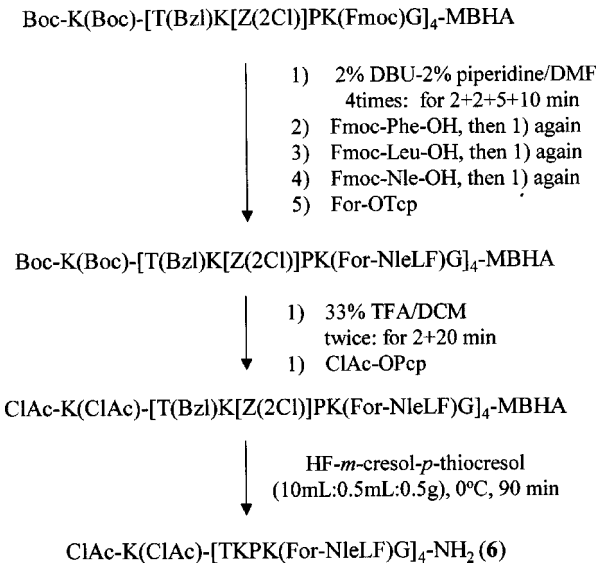

**Figure 1** Schematic structure of bioconjugate for chemotactic drug targeting (CDT).




**Scheme 1** Synthesis of branched oligopeptides with chemotactic tripeptides in the branches.

with liquid HF without any difficulties. In order to study the influence of charges on the chemotactic activity, we have prepared two further variants of Ac-[TKPK(For-MLF)G]$_4$-$NH_2$ (**1**). The free amino groups of the oligopeptide **1** were blocked by formylation to remove positive charges using For-OTcp in DMF (**4**). By succinylation of **1** with succinic anhydride (Scheme 1), we have produced compound **5**, which possesses negative charges instead of positive ones at the Lys$^2$ position. The succinylation was performed in 0.2 M $NH_4HCO_3$ solution at pH 6.5 to avoid the reaction with OH-group of threonine residues that can occur under alkaline condition. In this way, branched oligopeptides with positive (Ac-[TKPK(For-MLF)G]$_4$-$NH_2$ (**1**)), negative (Ac-[TK(Succ)PK(For-MLF)G]$_4$-$NH_2$ (**5**)) or no charges (Ac-[TK(For)PK(For-MLF)G]$_4$-$NH_2$ (**4**)) were prepared. However, the solubility of the compounds was decreased by the elimination of positive charges.

The next aim was to attach methotrexate to the carrier containing chemotactic moieties. To increase the cytotoxic activity of the conjugate, two copies of Mtx were incorporated. For this reason, the backbone was elongated by a Lys residue at the *N*-terminus. An enzyme labile spacer GFLG [19] was used to connect the drug and carrier molecules. According to the first trial, the GFLG spacer as well as Mtx was attached to the oligotuftsin backbone on the resin. However, the introduction of Mtx resulted in a derivative that was not stable under HF cleavage condition. A new strategy had to be developed for incorporation of Mtx into the carrier. First, methotrexate with the GFLG spacer elongated by Cys at the *C*-terminal was prepared on Rink Amide MBHA resin by Fmoc chemistry. This compound could




**Scheme 2** Synthesis of chloroacetylated chemotactic-branched oligopeptides.

be cleaved under mild acidic condition without decomposition of Mtx. Because unprotected methotrexate was used for the synthesis, both the $\alpha$- and $\gamma$-isomers were formed, and also the D-isomer of the $\alpha$-derivative – in accord with reported data [27] – was observed (Figure 2, Table 1). The compounds were isolated and identified according to Ref. 27. The Mtx-GFLGC-$NH_2$ was attached to the carrier via thioether bond formation. For this purpose, the chemoattractant carrier molecule containing chemotactic peptide For-NleLF in the branches was selected, and the *N*-terminal lysine residue was chloroacetylated with ClAc-OPcp at both $\alpha$- and $\varepsilon$-amino

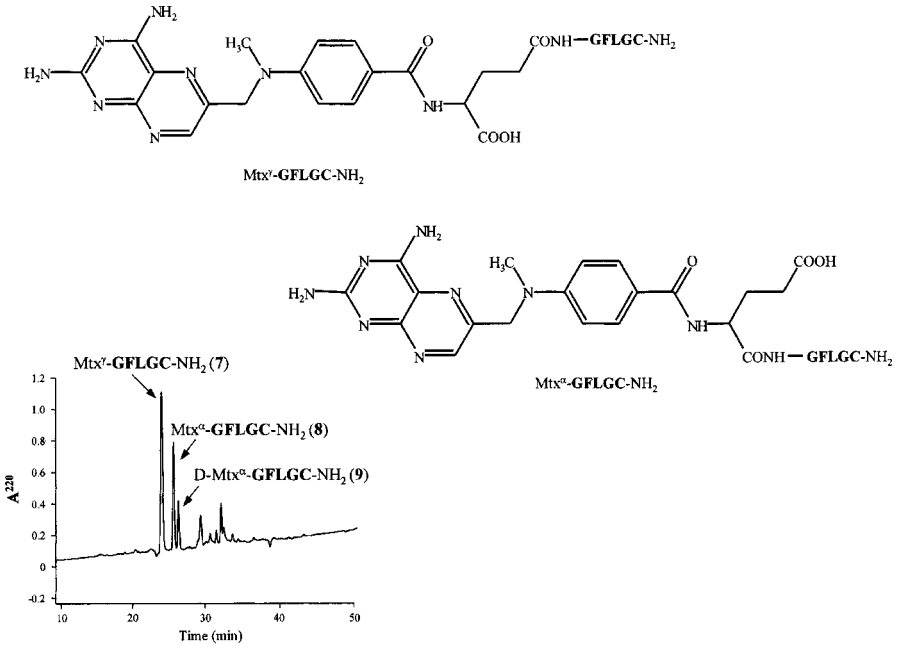


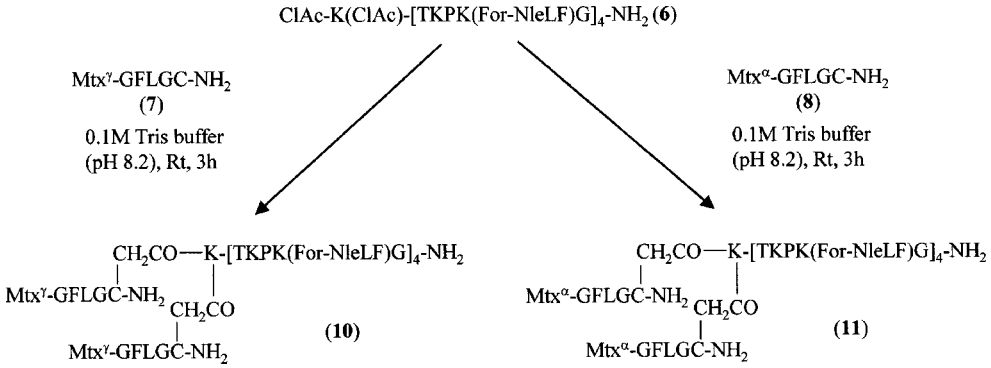

**Figure 2** Analytical HPLC chromatogram of crude Mtx-GFLGC-$NH_2$ (showing its corresponding structures).

groups resulting in compounds **6** (Scheme 2). Under a slightly alkaline condition, the chloroacetyl group reacts with the free thiol group of the Mtx-GFLGC-$NH_2$ forming thioether linkage between the spacer and the carrier. Both $^{\gamma}$Mtx-GFLGC-$NH_2$ and $^{\alpha}$Mtx-GFLGC-$NH_2$ were conjugated to the chemotactic carrier molecule resulting in compounds **10** and **11** respectively (Scheme 3). All compounds were purified by RP-HPLC and characterized by analytical HPLC, ESI-MS (Table 1) and amino acid analysis (data are not shown).

## Chemotactic Activity of the Conjugates with *N*-Formyl Tripeptides

Three different *N*-formyl peptides (For-MLF, For-NleLF, For-MMM) were conjugated to the tetratuftsin derivative. Comparison of the native chemotactic behaviour of the peptides with their conjugates shows significant differences. Conjugation of For-MLF to the tetratuftsin derivative results in significant loss of its chemotactic activity (Figure 3 (a) and (b)). However, by modifying the ionic charges of the carrier, the chemoattractant behaviour was regained. Tetratuftsin : For-MLF conjugate (**1**) with formyl or succinyl groups at the lysine residues exhibited higher activity than that of unmodified carrier. The chemotactic moiety, namely, For-MLF of the molecules proved to be sensitive to the charge properties of the carrier (Figure 3). The positively charged compound **1** turned to be a chemoattractant after formylation (**4**) in the $10^{-8}$–$10^{-6}$ M range, while in the lowest concentration tested ($10^{-12}$ M) this derivative had a slight chemorepellent character (Figure 3 (c)). Substitution with succinyl groups (**5**) shows a similar bi-phasic effect, but only two moderate peaks – a chemoattractant at $10^{-7}$ M and a chemorepellent at $10^{-11}$ M – were detected (Figure 3 (d)).




**Scheme 3** Synthesis of methotrexate-containing conjugates.

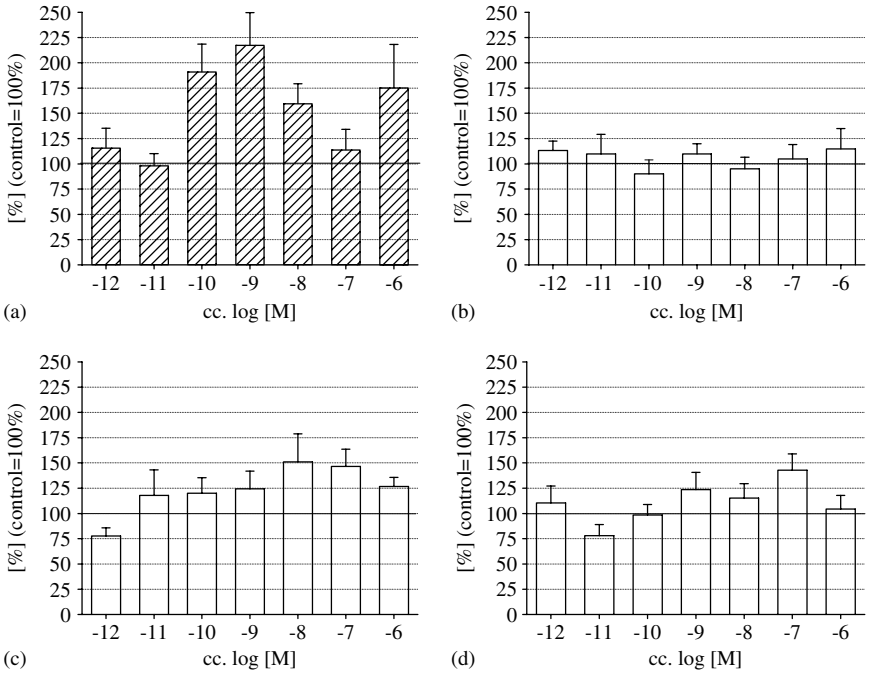



**Figure 3** Chemotactic response of *Tetrahymena pyriformis* cells induced by formyl-tripeptide (For-MLF) (a), its conjugate with tetratuftsin derivative (T20) (b), as well as formylated (c) and succinylated (d) derivatives of the conjugate.

The chemotactic tripeptide For-NleLF (Figure 4(a)) after the conjugation with the tetratuftsin derivative (Figure 4(b)) exhibited significantly elevated wide-range chemoattractant properties. It is interesting to note that the chemotactic activity of For-MMM did not change in its conjugate. This peptide and its conjugate exhibited neutral chemotactic activity in the concentration range investigated (data are not shown).

## Chemotactic Activity of Mtx Conjugates

The For-NleLF-containing conjugate was selected as the best chemoattractant compound for carrying methotrexate as a drug molecule. Both $\alpha$- and $\gamma$-methotrexate-bonded GFLGC spacers (**7** or **8**) were attached to the carrier, and their chemotactic behaviour was studied. Chemotactic responses elicited by the two conjugates (**10** and **11**) were different (Figure 5). Compound with Mtx attached to the carrier through the $\gamma$-amide bond (**10**) exhibited a bi-phasic character (Figure 5 (b)). It is chemoattractant at higher concentrations ($10^{-7}$–$10^{-6}$ M) and neutral or chemorepellent at lower concentrations ($10^{-9}$ and $10^{-12}$ M). In contrast, the $^{\alpha}$Mtx – For-NleLF conjugate (**11**) that possesses $\alpha$-amide bond–coupled Mtx elicits strong chemorepellent responses in the concentration range

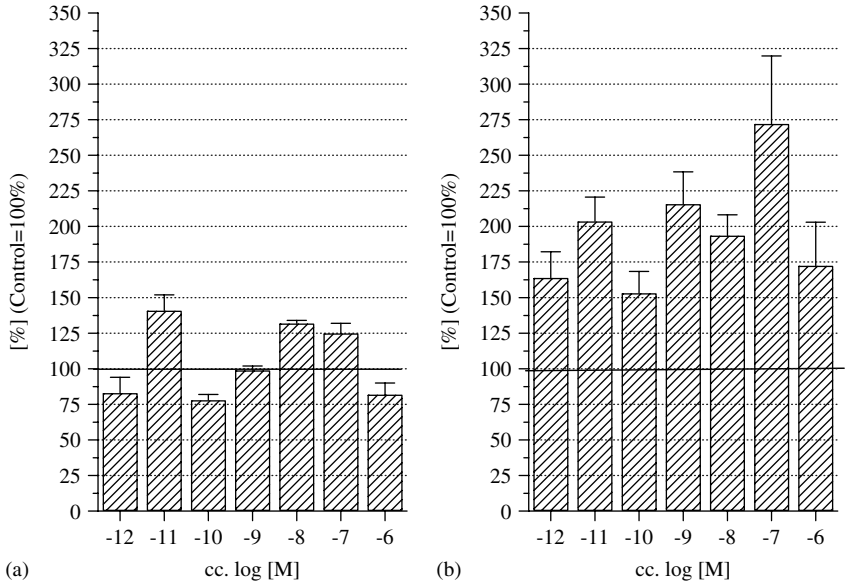



**Figure 4** Chemotactic response of *Tetrahymena pyriformis* cells induced by For-NleLF (a) or by its conjugate (b) with the tetratuftsin derivative (T20).

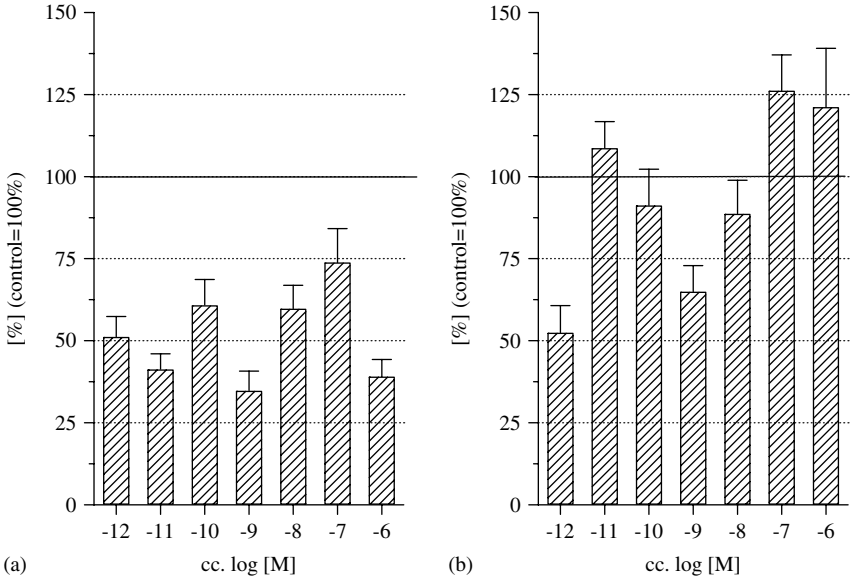



**Figure 5** Chemotactic activity of T20:For-NleLF conjugates with Mtx at the $\alpha$ (a) or $\gamma$ (b) position.

studied ($10^{-12}$–$10^{-6}$ M). These data suggest that not only the chemotactic part (For-NleLF) of the conjugate but also the Mtx moiety have a pronounced effect on the chemotactic properties.

### Cytotoxicity

In clinical protocols, the $10^{-6}$ M or higher plasma levels of Mtx are considered as (clinically) effective doses [28]. As distribution compartments of the body could significantly modify concentration of substances administered into the plasma, we have studied the cytotoxic effect of Mtx in $10^{-12}$–$10^{-6}$ M concentration range in *T. pyriformis* cells.

The cytotoxic effect of conjugates **10** and **11** was studied and compared with compound **2**, Mtx and $^{\gamma}$Mtx-GFLGC-$NH_2$ (**7**) as controls. We found that the two Mtx conjugates (**10** and **11**) are cytotoxic and exhibit a concentration-dependent profile with high level of similarity. At $c = 10^{-6}$ M, both conjugates like the free Mtx caused 70% cytotoxicity of *T. pyriformis* cells. These data clearly show that Mtx fully preserved its activity in the conjugates. The conjugate Ac-[TKPK(For-NleLF)G]$_4$-$NH_2$ (**2**) shows an almost negligible cytotoxic effect in the $10^{-7}$–$10^{-6}$ M range, while the $^{\gamma}$Mtx-GFLGC-$NH_2$ (**7**) ligand has only a moderate effect (Figure 6).

## CONCLUSION

Data on the chemotactic properties of a new class of the tetratuftsin derivative (T20)-based conjugates with For-XLF tripeptides suggest that T20 can be modified with For-NleLF to obtain a branched oligopeptide suitable for consideration as a carrier for CDT. We found that attachment of For-XLF to T20 can diminish (For-MLF) or elevate (For-NleLF) the chemoattractant capacity of formyl tripeptides. Data also indicate that charge properties of T20 also have a pronounced effect of the chemotaxis-induction of the conjugate. On the basis of those findings, the T20:For-NleLF compound was selected as a CDT carrier for coupling with Mtx. Cytotoxicity and chemotaxis results with these conjugates provided evidence that (i) Mtx preserved its cytotoxic activity in its covalently attached form and (ii) depending on the topology of Mtx coupling ($\gamma$ vs $\alpha$-amide bond), a chemoattractant conjugate can be produced. Studies with this compound in vertebrate model systems are in progress.

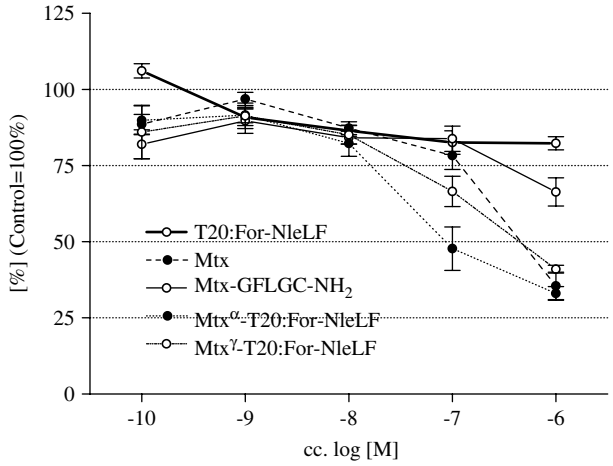



**Figure 6** Cytotoxic effects of Mtx-T20:For-NleLF conjugates and their components in *Tetrahymena pyriformis* cells.

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