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Efficient and chemoselective *N*-acylation of 10-amino-7-ethyl camptothecin with poly(ethylene glycol)

Andrea Guiotto,[a,*,†] Mirta Canevari,[a] Piero Orsolini,[b] Olivier Lavanchy,[b] Christine Deuschel,[b] Norimasa Kaneda,[c] Akinobu Kurita,[c] Takeshi Matsuzaki,[c] Takeshi Yaegashi,[c] Seigo Sawada[c] and Francesco M. Veronese[a,*]

[a]*Department of Pharmaceutical Sciences, University of Padova – via F. Marzolo, 5 35131 Padova, Italy*
[b]*DEBIO R.P., Route du Lèvant 146, CH-1920 Martigny, Switzerland*
[c]*Yakult Central Institute for Microbiological Research, Yaho 1796, Kunitachi-shi, Tokio 186, Japan*

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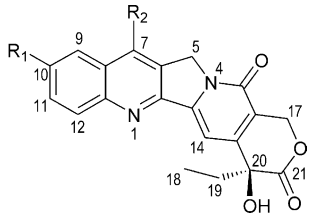
Abstract—A new poly(ethylene glycol) (PEG) conjugate of 10-amino-7-ethyl camptothecin, a potent antitumor analogue of camptothecin, has been synthesized and preliminary in vivo tests have been performed. Successful chemoselective *N*-acylation of 10-amino-7-ethyl camptothecin was accomplished using phenyl dichlorophosphate, a coupling reagent used in esterification of alcohols, while other coupling methods failed, due to the low nucleophilicity of the amino group in position 10. The conjugate was tested against P388 murine leukemia cell lines and resulted equipotent to CPT-11, a camptothecin analogue already in clinical use.

10-Amino-7-ethyl camptothecin (**1**, Fig. 1) has been synthesized by Yakult researchers[1] in a SAR research program aiming at developing new potent semi-synthetic anticancer analogues of camptothecin (CPT, **2**) modified at the aromatic A ring. Substitutions at positions 9, 10, 11 and 12 of the benzene ring are known to modulate the drug pharmacological parameters,[2] and the presence of an amino group in position 9 or 10 has been shown to increase activity, although a substantial inactivation of the agent due to unfavorable lactone/carboxylate ratio in humans[3] halted further studies of phase II trials. The preparation of poly(ethylene glycol) (PEG) prodrugs of CPT[4] and 10-hydroxy camptothecin (10-HCPT)[5] has been reported, in order to obtain water soluble prodrugs with longer retention times, selective accumulation at tumor site due to the EPR (Enhanced Permeability and Retention) effect, and protection of the active lactone form. We wish to report here the high yield, chemoselective synthesis of a PEG conjugate of 10-amino-7-ethyl camptothecin with a procedure only recently reported by Greenwald et al. for 10-HCPT.[5]

Within a wider research project aiming at the production of PEG conjugates of 10-amino-7-ethyl camptothecin, we faced the need for a simple, chemoselective acylating step converting (**1**) into the desired PEGylated derivative **3**, without the need of a lengthy protection/deprotection protocol for the 20-OH moiety (Scheme 1). Although in principle the selective acylation of the 10-amino group should be straightforward, since the 20-hydroxyl group is a highly hindered aliphatic tertiary alcohol, standard coupling methods derived from peptide synthesis (carbodiimide + tertiary base, with or without HOBt) failed to produce the desired PEGylated product. A variety of reaction conditions, including bases such as triethylamine, dimethylaminopyridine



1, $R_1 = NH_2$, $R_2 = Et$
2, $R_1 = R_2 = H$

Figure 1. Structures of 10-amino-7-ethyl camptothecin (**1**) and camptothecin (**2**).

Keywords: PEG; Camptothecin; Conjugation; Phenyl dichlorophosphate.
* Corresponding authors. Fax: +39-049-8275660; e-mail: andrea.guiotto@unipd.it; francesco.veronese@unipd.it
† Present address: C.N.R. Institute of Biomolecular Chemistry, Padova Unit, via F. Marzola, 1 35131 Padova, Italy.

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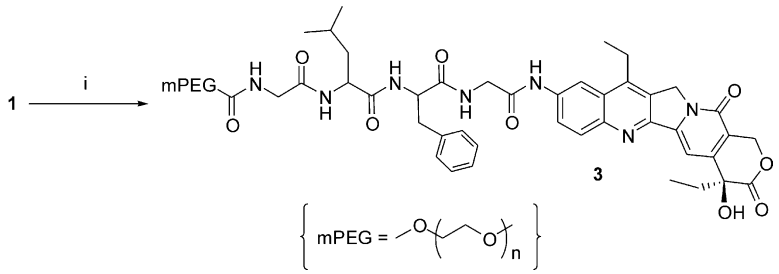

Scheme 1. Reagents and conditions: (i) PEG-Gly-Leu-Phe-Gly-OH, phenyl dichlorophosphate, pyridine, *i*-Pr_2NEt; 12 h, rt.

(DMAP), diisopropyl ethylamine (*i*-Pr_2NEt, Hunig's base) and excess of reagents (up to 4 equiv of **1** and coupling reagents) only resulted in low/medium yields of 10-acylated product, together with other undetermined PEGylated products (probably, 20-*O*-mono-PEGylated and 10, 20-diPEGylated derivatives) detected by C18HPLC analysis, but difficult to separate from the desired compound (Table 1). Interestingly, the model reagent acetyl-Phe-OH used to test the reaction conditions completely failed to couple to **1** (Table 1, entries 1 and 2), probably due to steric hindrance. First reported by Liu and colleagues[6] for the esterification of alcohols, the phenyl dichlorophosphate coupling reagent was used to produce 20-*O*-PEGylated CPT derivatives in a U.S. Patent issued to Enzon.[7] Similar reagents (diphenylphosphinic acids) are known in peptide synthesis[8] to form a phenylphosphoric–carboxylic acid anhydride active species, promptly converted into an amide by reaction with the appropriate amine, but to our knowledge no literature reference reported so far the use of phenyl dichlorophosphate in amide formation, nor its possible chemoselectivity towards amino groups in presence of unprotected hydroxyl groups.

Table 1. Acylation of **1**: reaction conditions and yields

Entry	Conditions (equivalents[a])	Yield (%)[b]
1	Acetyl-Phe-OH, EDC, DMAP (1,5:1,6:1,6); CH_2Cl_2	0
2	Acetyl-Phe-OH, EDC, DMAP (3:3:3); CH_2Cl_2	0
3	**1**, EDC, *i*-Pr_2NEt (2:2:2); CH_2Cl_2	45
4	**1**, EDC, HOBt, *i*-Pr_2NEt (4:4:4:4); CH_2Cl_2	50
5	**1**, PhOP(=O)Cl_2, pyridine, DMAP (cat.); $CHCl_3$	95[c]

[a] Equivalents with respect to PEG-Gly-Leu-Phe-Gly-OH.
[b] Loading of **1** (w/w) calculated from UV titration curve and HPLC.
[c] Mean value of 3 experiments.

Table 2. In vitro data of PEG 10-amino-7-ethyl camptothecin (**3**) derivative

Sample[a]	Total dose (mg/kg)[b]	Survival time (days, mean ± SD)	T/C (%)[c]
Derivative **3**	20[d]	13.0 ± 1.1	170
CPT-11	25	13.2 ± 1.7	172
Adriamycin	25	> 14.0 ± 8.7	> 183
Control	—	7.7 ± 0.5	100

[a] In vivo tests were run on murine leukemia P388 cells. Female CDF1 mice were inoculated intra-peritoneally with P388 at dose of 1×10^6 cells/mouse on day 0, and injected intravenously with the derivative on days 1, 5 and 9.
[b] Derivatives were injected intravenously on days 1, 5 and 9 at total doses indicated and survival times were monitored for 40 days.
[c] Survival rate (T/C%) was calculated using the following formula: T/C% = (mean survival days of treated group/meal survival days of control group) × 100.
[d] Dose equivalent of free 10-amino-7-ethyl camptothecin.

Using phenyl dichlorophosphate we successfully acylated with high and reproducible yields the 10-amino camptothecin derivative **1** at the amino group, providing evidence of the applicability of such procedure to amino-containing compounds as suggested, but not reported, by Greenwald et al. in a recent publication.[5] The same authors confirmed the 10-acylation by ^1H NMR data (20-acylated camptothecin derivatives exhibit C-19 protons as two quartets compared to the un-acylated camptothecin as a single quartet). Similarly, the selective acylation was confirmed in our molecule by ^1H NMR (single quartet at 1.89 ppm for the C-19 protons of the N-acylated product **3**). In vitro data for compound **3** using P388 cell line (Table 2) show an activity comparable to CPT-11 (Irinotecan®), a camptothecin analogue already in clinical use for treatment of colorectal cancer.

In conclusion, the difficult acylation of the amino group of 10-amino-7-ethyl camptothecin (**1**) has been successfully performed without concurrent formation of the undesired C10 ester. Phenyl dichlorophosphate, reported in literature as a reagent for the esterification of alcohols, has been here successfully employed for the *N*-acylation in presence of an unprotected tertiary alcohol. It is noteworthy that the acylation of a poorly reactive aniline could be achieved without the need for strong reagents (i.e., acyl halides) incompatible with the reaction substrates. The PEGylated 10-amino-7-ethyl camptothecin **3** was obtained in high yield and purity[9] and was comparable to CPT-11 in preliminary in vitro tests against P388 murine tumor cell line.

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9. **Preparation of mPEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Phe-Gly-10-amino-7-ethyl-camptothecin** (**3**). 0.6 g (0.06 equiv) of mPEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Phe-Gly-OH and 45 mg (0.12 mmol, 2 equiv) of 10-amino-7-ethyl camptothecin were dissolved in 30 mL of toluene and the mixture was azeotropically distilled with removal of 25 mL of toluene. The mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 30 mL of dry chloroform. Pyridine (0.24 mL, 3 mmol) and phenyldichlorophosphate (0.35 mL, 2.28 mmol) were added and the mixture was stirred at room temperature for 12 h, after addition of a catalytic amount of *i*-Pr$_2$NEt. At the end, the yellow mixture was extracted with 1N HCl (20 mL). The organic phase was concentrated at reduced pressure and the residue, diluted with 20 mL of 2-propanol, was re-crystallized. The pale yellow crystalline precipitate was washed with diethyl ether affording 0.45 g of pure product (**3**). ^{1}H NMR ($CDCl_3$) ppm: δ 0.83–0.91 (m, 6H, Hδ Leu); 1.03 (t, 3H, CPT 18-H); 1.26 (m, 1H, Hγ Leu); 1.42 (t, 3H); 1.61 (m, 2H, Hβ Leu); 1.89 (q, 2H, CPT H-19); 3.05 (d, 2H, Hβ Phe); 3.19 (m, 2H, CPT); 3.38 (s, 3H, PEG –OCH_3); 3.39–3.89 (m, PEG + Hα Gly); 4.17 (m, 1H, Hα Leu); 4.26 (t, 1H, Hα Phe); 5.24 (s, 2H, CPT H-5); 5.29 (d, 2H, CPT H-17); 5.72 (m, 1H, –O(CO)NH–); 6.5 (bs, 1H, 20-OH); 7.24–7.30 (m, 8H, Arom.); 7.7 (s, 1H, CPT H-14); 8.18 (s, 1H); 8.35 (s, 1H); 8.90 (bs, 1H); 9.50 (s, 1H). RP-HPLC: Alltima C18 analytical column 150×4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA), flow rate 1 mL/min, on-line UV detector at 368 nm. Mobile phase A: milliQ grade water, 0.05% TFA; B: acetonitrile, 0.05% TFA. Gradient: Time (% B): 0 (10%), 30′ (80%), 36′ (10%). Single peak eluting at 18.2 min. Maldi-Tof mass spectroscopy (sinapinic acid matrix): M^+ centered at 10765 Da. UV (methanol): maxima at 382, 368, 265 nm (10-amino-7-ethylcamptothecin maxima at 400, 320, 260 nm; PEG-Gly-Leu-Phe-Gly-OH maxima at 265 nm). The amount of 10-amino-7-ethyl-camptothecin (**2**) determined in the obtained derivative was 16.7 mg, corresponding to a w/w% of 3.71% (the theoretical 100% of loading is, for this conjugate, 3.72% according to UV absorption).