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Tripartate Poly(ethylene Glycol) Prodrugs of the Open Lactone Form of Camptothecin

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Abstract—Two PEG prodrugs utilizing conjugation of PEG through the C-21 acid functionality as well as the C-17 OH group of CPT hydroxy-amide open forms were synthesized and characterized. Both of these open lactone tripartate prodrugs were shown to be water soluble and highly effective in MX-1 mouse xenograph studies. Indirect evidence implies that the initial ester or carbonate bond breaking is esterase mediated in the first step of the cascade of CPT release.

Introduction

It has been definitely established that an intact δ-lactone (ring E) must be present for full expression of the in vivo anti-tumor activity of camptothecin (CPT, 1) and related synthetic CPT derivatives,[1] which are potent anticancer agents.[2] The sodium salt of the open form of the lactone, **1a**, is water soluble and shows in vitro activity and inhibition of topoisomerase I comparable to **1**.[3] A similar situation has been reported for 10-hydroxy CPT.[4] However, clinical evaluation of **1a** was not successful, and it was later claimed that **1a** is only 10–20% as active as **1**.[5] It is likely that in human trials **1a** is slowly converted into **1** in serum and that any differences observed for in vivo efficacy reflects the different pharmacokinetic properties of the two forms.[3] No further clinical candidates of lactone open forms of CPT derivatives have been developed.

In 1979, Adamovics[1] demonstrated that ring opened amide derivatives of CPT were stable enough to isolate and acylate, yielding more stable ester-amide derivatives. These compounds demonstrated poor in vitro activity in cell tissue culture experiments. However, comparable in vivo efficacy to **1** was demonstrated in mice using P388, L1210, and B16 tumors. Sawada[6] also confirmed that for 7-ethyl-camptothecin, ring opening of the lactone ring with diamines produced basic hydroxy-amides which could be acylated, and which were water soluble as their hydrochloride salts. These derivatives were shown to be more active than the salt **1a** in L1210 inoculated mice. More recent investigations have further verified these findings.[4,7] Thus, CPT open amide-ester derivatives function as tripartate prodrugs where the rate determining step of the conversion is dependent on two factors: initial hydrolysis of the ester followed by ring closure to the active CPT lactone derivative.[1] This property has the potential to be developed into clinically relevant CPT derivatives that to our knowledge are non-existent. Our interest in using polyethylene glycol (PEG) to prepare 20-ester prodrugs[8,9] has led to the development of Prothecan® (**15**) which is currently in Phase II clinical trials.[10] It naturally followed that the potential of open PEG-CPT amide-ester prodrug forms should be evaluated in order to determine if these compounds offered a more effective route for drug delivery of CPT. In this work, we describe our efforts to prepare PEGylated versions of CPT open forms, and report on their in vitro and in vivo properties (Fig. 1).

Chemistry

The first PEGylated open CPT was a hydrolytically reactive carbonate prepared by employing the known dimethylaminoethyl amide derivative, **3**.[6,7] Condensation of **3** with the activated bifunctional spacer unit **4** in the presence of dimethylamino pyridine (DMAP) gave the Boc protected carbonate **5**, which was easily deprotected using

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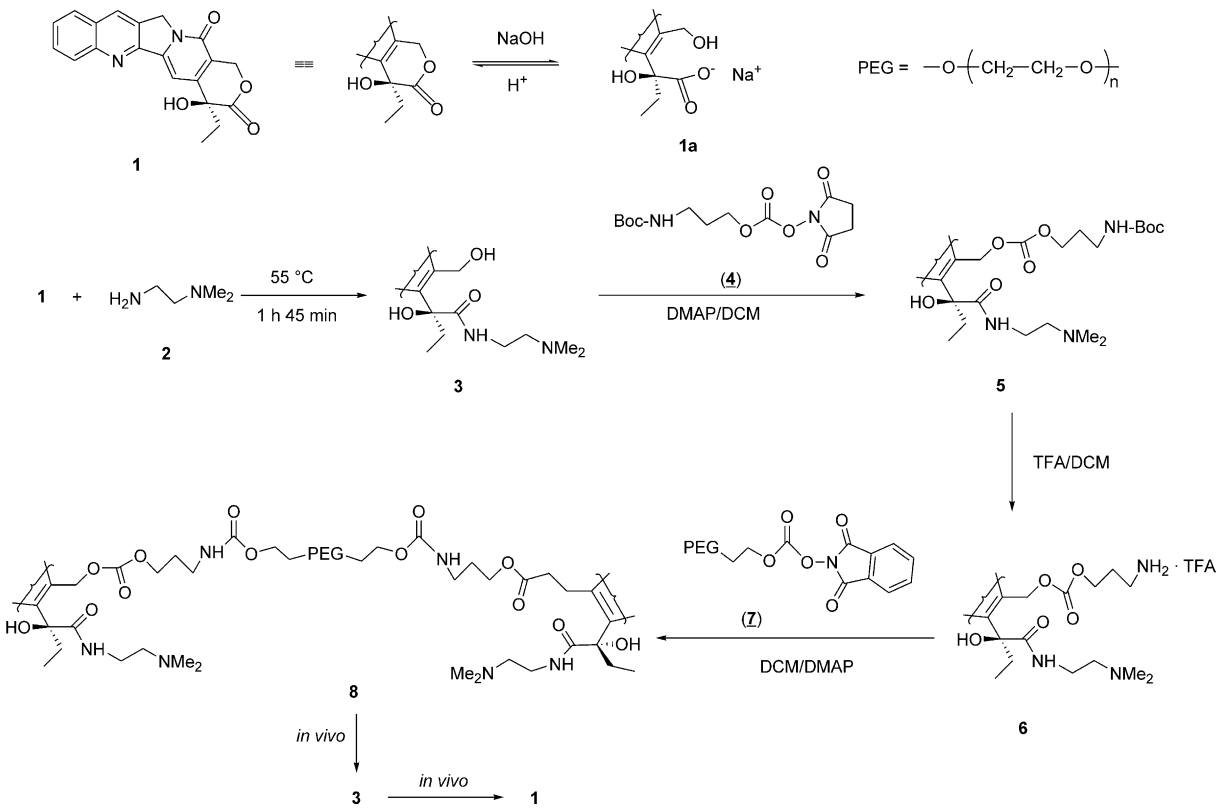

Scheme 1.

trifluoroacetic acid (TFA) to the free amine **6**. Pegylation of **6** with PEG (40,000) bis-phthalimido carbonate (**7**) produced the desired di-substituted open CPT prodrug **8** in 80% yield (Scheme 1). Another modification of prodrug design which utilized conjugation of PEG through the C-21 acid functionality rather than the C-17 OH group was done as shown in Scheme 2, using the mono Boc protected diamine **9**.[11] Again **1** was conveniently ring opened to give the open amide derivative **10**. This could be blocked by any activated acid, but acetic anhydride was chosen for convenience and gave **11** in reasonable yield (40%). TFA deprotection followed by facile condensation of **12** with the activated PEG carbonate **7** gave the novel PEG prodrug **13**.

Results

The efficacy of open-form camptothecin analogues against a subcutaneous human mammary carcinoma (MX-1) grown in nude mice was determined as follows. Following at least one week of acclimation, tumors were established by implanting small tumor fragments from donor mice into a single subcutaneous site, on the left axillary flank region of nude mice. The tumor implantation site was observed twice weekly and measured once palpable. The tumor volume for each mouse was determined by measuring two dimensions with calipers and calculated using the formula: tumor volume = (length × width^2)/2. When tumors reached the average volume of approximately 75 mm^3, the mice were divided into their experimental groups, which consist of untreated controls, Prothecan® (PEG-Ala-Camptothecin) (**15**), **8**, and **13**. The mice were sorted to evenly distribute tumor size, grouped into 4–5 mice/cage, and ear punched for permanent identification. Drugs were dosed intravenously via the tail vein as a single dose (qd × 1). Mouse weight and tumor sizes were measured at the beginning of study and twice weekly through week 5.

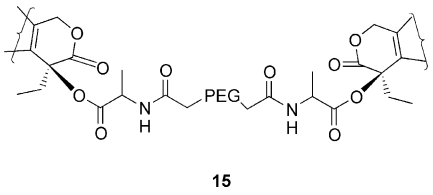

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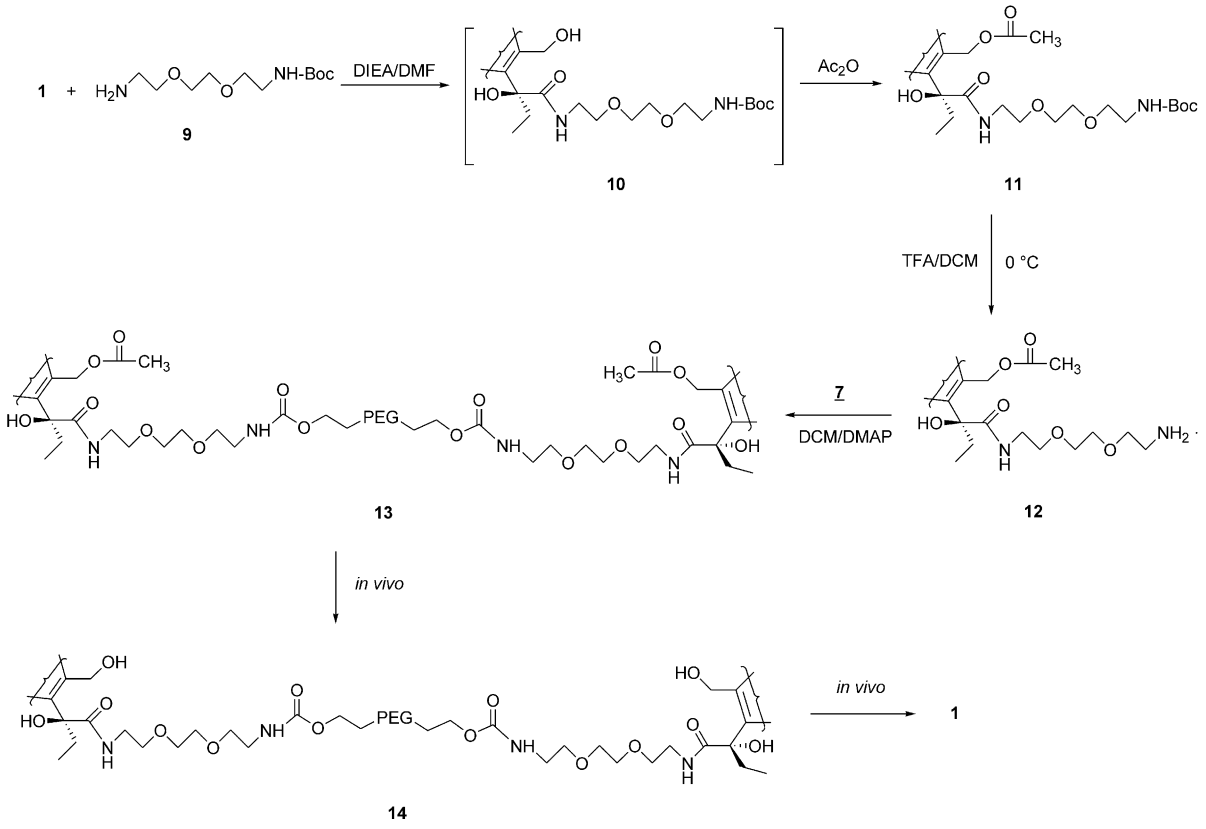
Figure 1.

The overall growth of tumors was calculated as the mean tumor volume at 1 week following the end of the treatment. A percent treatment over control (T/C) value was also calculated when the control group's median tumor size reached approximately 800–1100 mm^3 and again at 1 week following treatment. The T/C value in percent is a non-quantitative indication of anti-tumor effectiveness.

The in vitro properties of **8** and **13** were obtained as previously described for **15**,[9] and are shown in Table 1. The prodrugs are quite stable in aqueous saline indicating that dissolution of these compounds prior to use will provide stable formulations. Half-lives in rat plasma are



Scheme 2.

on the order of, or slightly higher than the cyclized prodrug, **15**, which are indicative of the residence time of 40,000 molecular weight PEG and the stability of the connecting bond to drug. Treatments with **8**, **13**, and **15** all caused significantly ($p < 0.05$) smaller tumor volumes as compared to control mice. In addition, at equivalent doses, there was no significant ($p < 0.05$) difference in the antitumor activity of these three compounds (Table 2).

Discussion

The results of the in vitro and in vivo testing upon close examination are quite informative. Clearly native CPT and **15**, a 20-ester PEG prodrug lactone form of CPT, are extremely cytotoxic towards P388 murine leukemia cells in cell tissue culture experiments (Table 1). On the other hand, prodrugs **8** and **13** show little efficacy in tissue culture. Since esters and carbonates generally cleave in the cell tissue culture media,[12] it is likely that the prodrugs have cleaved to their respective open hydroxy acid forms, for example **3** in the case of **8**, and **14** in the case of **13**, both of which are not active.[1] Similar results were obtained by Adamovics[1] for an acetate-*iso*propyl amide prodrug using L1210 cells, indicating that the open hydroxy amide (not active) had been generated. These compounds have been isolated,[1,6,7] and can be expected to have enough stability to survive cell tissue culture experiments. Thus, bipartate prodrug hydrolysis appears to be relatively rapid, and therefore cyclization (second step of the tripartate system) is the rate determining step in the generation of the lactone species. The fact that any in vitro cytotoxicity was observed in our findings is probably due to the presence of a small amount of cyclized (active) lactone species being present. By contrast, our findings with respect to the in vivo results of comparing all compounds in an MX-1 mouse xenographed tumor model (Table 2) provide data that demonstrate not only the feasibility of using PEG prodrug open forms of CPT, but the EPR effect as well,[13] leading to greater amounts of drug accumulation in the tumor. Compared to the negative in vitro results, in vivo testing shows **8** and **13** to be equivalent to the closed lactone ring derivative **15**. Therefore, cyclization of the hydroxy amide must be occurring in vivo, and since it has been established that with PEG 40,000 derivatives tumor accumulation occurs to a greater extent than **1** alone,[9] it follows that the open forms **3** and **14** are generated after passive accumulation in the interstitial space of the tumor followed by lactonization in the acidic environment of the tumor.[14] Thus the PEG-prodrug approach may reduce overall toxicity and enhance the therapeutic index of CPT.

Conclusions

Retrospection of earlier work describing derivatives of open lactone forms of CPT has verified their in vivo effectiveness compared to the closed form in animal models. Further modification of **1** was carried out to incorporate a high molecular weight PEG on two different functionalities of the open lactone ring, both of

Table 1. Properties of PEG derivatives of camptothecin open forms

Compd	$t_{1/2}$ (h) (PBS, pH = 7.4)	$t_{1/2}$ (h) (rat plasma)	Solubility in saline[a] (mg/mL)	IC_{50} (P388, nM)
1	—	—	0.0025	7
8	>48	21	2.79	678.3
13	>48	34	2.34	1190
15	>48	15	~2.0	16

[a]Based on camptothecin.

Table 2. Summary of PEG-camptothecin efficacy against a human mammary carcinoma (MX-1) xenograft in nude mice[a]

Compd	Tumor volume (mean ± SEM) day 21	T/C (%)[b] at 1000 mm^3	Tumor regression at day 25[c] (#/grp)
Control	2722 ± 223	—	0/5
15	15 ± 5	1.4	5/5
8	21 ± 6	3.0	5/5
13	14 ± 2	2.5	4/4

[a]Intravenous treatment in nude mice bearing established tumors (~75 mm^3). $N = 4$–5/group. The compound was injected via a single dose at 24 mg/kg equivalent to camptothecin.
[b]The median tumor volume of treatment and control groups were measured and compared when the control group's median tumor volume reached approximately 1000 mm^3 and one week after the dosage (day 15). T/C < 42% at 1000 mm^3 is considered significant anti-tumor activity by the Drug Evaluation Branch of the NCI.
[c]Complete tumor regression was observed in all mice except the ones in the control group.

which, using a mouse model, demonstrated in vivo efficacy equivalent to the PEG conjugated CPT derivative (**15**) already in clinical trials. The value of these PEG prodrug systems is two-fold: they enable aqueous solutions to be prepared easily and thus solve a difficult formulation problem, one encountered for most CPT derivatives. In addition the PEG ballast provides passive tumor accumulation, which enables the tripartate prodrug sufficient time to accumulate and decompose in the tumor stroma.

Experimental

General procedures

All reactions were run under an atmosphere of dry nitrogen or argon. Commercial reagents were used without further purification. CPT was obtained from Boehringer-Ingelheim. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use. ^{13}C NMR spectra were obtained at 75.46 MHz using a Varian Mercury® 300 NMR spectrometer and deuterated chloroform as the solvent unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Mass Specteral data was obtained from the Biotechnology Research Lab at Yale University. All PEG conjugated compounds were dissolved in saline for injection prior to in vivo drug treatments and were given as their camptothecin equivalents (absolute amount of camptothecin given).

HPLC methods

The reaction mixtures and the purity of intermediates and final products were monitored by a Beckman Coulter System Gold® HPLC instrument employing a Zorbax® 300 SB C-8 reversed-phase column (150×4.6 mm) or a Phenomenex Jupiter® 300A C-18 reversed-phase column (150×4.6 mm) with a multiwavelength UV detector monitored at 360 nm, using a gradient of 30–90% of acetonitrile in 0.05% TFA at a flow rate of 1 mL/min.

Compound 3. Camptothecin (**1**) (1.44 g, 4.14 mmol) was dissolved in *N,N*-dimethyl-2 aminoethane, and **2** (15 mL) added. The reaction solution was heated at 50 °C for 2 h, the solvent removed under reduced pressure, and the resulting solid washed with ether to give **3**. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 173.57, 161.50, 154.08, 152.53, 150.15, 148.51, 143.05, 130.96, 130.51, 129.71, 129.40, 128.35, 127.94, 127.45, 118.68, 98.06, 78.91, 77.21, 62.46, 50.05, 36.65, 31.22, 7.91. MS (ES^+) m/z 437 $[M+H]^+$.

Compound 5. A solution of compound **3** (0.97 g, 2.22 mmol), dimethylaminopyridine (DMAP, 1.0 g, 8.88 mmol), and compound **4** (1.3 g, 4.44 mmol) in anhydrous methylene chloride (DCM, 10 mL) was refluxed for 4 h and then stirred at room temperature for 12 h. The reaction mixture was washed with 0.1 N HCl, dried with $MgSO_4$, filtered and the solvent evaporated under reduced pressure. The crude material was purified using silica gel chromatography to give **5** (0.80 g, 60%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 167.26, 157.15, 155.77, 153.69, 152.18, 148.76, 146.33, 145.71, 131.14, 130.65, 129.56, 128.37, 128.10, 128.02, 120.10, 95.82, 79.14, 77.83, 77.20, 67.01, 66.50, 50.03, 36.97, 31.84, 29.01, 28.37, 25.59, 7.73.

Compound 6. To a solution of compound **5** (0.080 g, 0.13 mmol) in DCM (1 mL) cooled in an ice bath, TFA (1 mL) was added drop-wise with stirring over 20 min. The solvents were removed under reduced pressure to give **6** (0.080 g, 0.13 mmol, ~100%). ^{13}C NMR

(67.8 MHz, $CDCl_3$) δ 167.50, 157.01, 153.95, 146.42, 145.31, 143.81, 134.69, 132.80, 129.26, 129.08, 128.52, 126.56, 120.92, 98.92, 78.27, 77.21, 66.69, 65.27, 50.47, 37.15, 31.33, 26.55, 25.58, 7.53.

Compound 7. A solution of 40 kDa PEG diol (5.0 g, 0.13 mmole) in toluene (75 mL) was azeotroped for 2 h under a nitrogen atmosphere while removing 25 mL of toluene/water. This solution was cooled to 30 °C, followed by the addition of triphosgene (49 mg, 0.17 mmol) and pyridine (40 μL, 0.5 mmol). The resulting mixture was quickly heated to 50 °C and maintained at that temperature for 1 h, then *N*-hydroxyphthalimide (0.10 g, 0.63 mmol) and pyridine (50 μL, 0.63 mmol) were added to the reaction and stirring continued at 50 °C for an additional 20 h. The solution was filtered at 50 °C and the solvent evaporated under reduced pressure. The residue was crystallized from a mixture of DCM (20 mL) and ethyl ether (80 mL) to yield **7** (4.3 g, 0.11 mmol, 86%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 160.82, 151.98, 134.57, 128.12, 123.61.

Compound 8. A solution of **6** (0.080 g, 0.13 mmol), **7** (1.50 g, 0.037 mmol), and DMAP (0.031 g, 0.25 mmol) in DCM (8 mL) was stirred at room temperature for 12 h. The reaction mixture was washed with 0.1 N HCl (2×10 mL) and the organic layer was evaporated under reduced pressure. The solid residue was crystallized from isopropyl alcohol (IPA, 30 mL) to yield **8** (1.2 g, 0.029 mmol, 79%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 166.41, 156.36, 155.64, 152.92, 151.50, 148.01, 145.76, 144.89, 130.76, 129.90, 128.84, 127.95, 127.58, 127.29, 119.31, 94.90, 66.29, 65.78, 63.05, 49.46, 36.69, 31.12, 28.39, 7.14.

Compound 11. A mixture of **1** (0.34 g, 0.97 mmol), **9** (4.7 g, 19 mmol), and diisopropylethylamine (DIEA, 3.3 mL, 1.4 mmol) in dimethylformamide (DMF, 7 mL) was heated at 70 °C for 48 h and a clear solution was obtained. The solvent was removed under reduced pressure, and the resulting solid washed with hexane and then ether to give **10** which was suspended in anhydrous DCM (10 mL) and cooled to 0 °C in an ice bath. Acetic anhydride (0.10 mL, 1.1 mmol) and DMAP (0.057 g, 0.47 mmol) were added to the above suspension and stirred for 3 h at ambient temperature. The reaction mixture was washed with 0.1 N sodium bicarbonate (10 mL), and the organic layer evaporated under reduced pressure to yield a solid product. The residue was purified on silica gel column to give pure **11** (0.25 g, 0.39 mmol, 40%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 172.19, 170.95, 161.38, 156.80, 155.91, 151.42, 148.04, 144.05, 130.42, 130.00, 128.92, 127.89, 127.68, 127.58, 127.26, 124.77, 100.60, 79.15, 78.55, 70.14, 69.37, 58.78, 50.23, 40.33, 39.04, 32.93, 28.40, 20.99, 7.92. MS (ES^+) m/z 661 $[M+Na]^+$.

Compound 12. To a solution of compound **11** (0.12 g, 0.19 mmol) in DCM (1 mL) cooled in an ice bath was added TFA (1 mL) drop wise with stirring over a 30 min period. The solvents were removed under reduced pressure to give 12 (0.12 g, 0.19 mmol, ~100%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 173.01, 172.22, 161.33, 157.08, 149.97, 146.13, 142.92, 132.94, 131.47, 128.55, 128.40, 128.10, 127.67, 127.23, 125.13, 117.41, 113.59, 102.62, 78.65, 70.19, 69.37, 66.48, 58.63, 50.67, 40.06, 39.28, 32.68, 20.78, 7.62.

Compound 13. A solution of **12** (0.050 g, 0.090 mmol), **7** (1.20 g, 0.030 mmol), and DMAP (0.022 g, 0.18 mmol) in DCM (6 mL) was stirred at room temperature for 12 h. The reaction mixture was washed with 0.1 N HCl (2×10 mL) and the organic layer evaporated under reduced pressure. The solid residue was crystallized from IPA (30 mL) to yield **13** (1.1 g, 0.027 mmol, 89%). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 171.82, 170.32, 160.71, 155.75, 155.23, 151.59, 147.82, 143.79, 134.41, 130.30, 129.55, 128.70, 127.90, 127.42, 127.11, 124.07, 99.59, 63.14, 58.50, 49.61, 40.17, 38.58, 32.14, 20.48, 7.38.

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