Single-Step Synthesis of Cell-Permeable Protein Dimerizers That Activate Signal Transduction and Gene Expression

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Abstract: The one-step "dimerization" (with loss of ethylene) of the immunosuppressant FK506 by olefin metathesis has been used to create new chemical inducers of dimerization (CIDs). These small-molecule protein dimerizers are shown to activate signal transduction pathways and gene transcription at low concentrations in mammalian cells. Their ease of synthesis provides ready access to reagents of widespread utility in studies of the cellular functions of proteins.

Introduction

Rationally designed small-molecule ligands containing two protein-binding surfaces have provided a general method to activate many cellular processes, especially signal transduction pathways. Thus far, these molecules have been used to activate proteins consisting of natural functional domains fused to immunophilin dimerization domains. This method has proven to be general, providing means to control with small molecules conditional variants of the cell surface T cell[1] and Fas receptors,[2] the intracellular proteins Src,[3] SOS,[4] and Raf,[5] and numerous transcriptional activators,[4,6] and it has been used to translocate intracellular proteins to the plasma membrane[4−6] and to the nucleus.[4] The method has also been shown to be highly effective in animals, allowing the cell-specific activation of a cell death signaling pathway in mice, the induction of mesoderm tissue in frog embryos,[7] and the *in vivo* regulated production of human growth hormone in mice.[8]

Many of the above processes were activated by molecules produced in multistep syntheses involving the attachment of linking elements to the natural protein dimerizers that inspired these studies, FK506, rapamycin, and cyclosporin. The complexity of these syntheses places limitations on the routine availability of these reagents, especially when used to regulate biologic pathways in animals. We now report a one-step synthesis of a chemical inducer of dimerization (CID) that produces ready access to a nontoxic CID that potently activates specific signaling pathways in cell culture and in animals. This concise synthesis produces biologically useful chemical inducers of dimerization in a single step without functional group protection. The potencies of the new compounds obtained by this method are contrasted with a previously reported CID[1a] FK1012A (Scheme 1).

Results and Discussion

FK506 **1** was dimerized (with loss of ethylene) by olefin metathesis[9] without protection of the potentially coordinating alcohol functionality at C24 or C32. When subjected to catalytic amounts of $(Cy_3P)_2Cl_2Ru{=}CHPh$[9a] (**2**) in dichloromethane at ambient temperature under argon, **1** (0.05 M) was converted to a less polar compound (tlc). After 22 h, the non-immunosuppressive dimeric product **3** was isolated in 49% yield as a *ca*. 1:1 mixture of alkene isomers after silica gel chromatography. The balance of the mass was unreacted FK506 **1** and a trace byproduct, identified as the C39 benzylidene derivative of FK506 by FAB-MS. It proved possible to increase the yield of **3** (58%) by increasing the concentration of **1** in the presence of 5 mol % **2** and replenishing the catalyst (5 mol %) once during the reaction.

Because useful levels of stereoselectivity could not be coaxed from the metathesis,[10] the product alkenes **3** were separated by chromatography (Ag^+ impregnated SiO_2).[11] Due to obscured olefin resonances and geometric isomerism, the stereoisomers **3E** and **3Z** were assigned on the basis of a comparison of the ^{13}C chemical shifts of the allylic (C38) and olefinic (C39) carbons obtained by an inverse-detected $^1H-^{13}C$ HMQC experiment. The chemical shift of C38 in **3Z** (*C38*, δ 28.9; *H38*, δ 2.48) was found to be 5 ppm upfield of C38 in **3E** (*C38*, δ 33.9; *H38*, δ 2.36). Furthermore, the olefinic carbon (C39) of **3Z** (δ 127.9) appears *ca*. 1 ppm upfield of that of **3E** (δ 129.2).[12]

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(10) The stereoselectivity of the metathesis proved relatively insensitive to solvent effects and temperature. However, modest *E*-selectivity (4:1 *E/Z*) could be achieved by conducting the "dimerization" in refluxing *t*-BuOH (23% isolated yield). Heating in DCM or DCE did not affect the selectivity. Improved *Z*-selectivity (3:1 *Z/E)* could be obtained by TBS protection of the C-24 hydroxyl group. Efforts to interconvert the geometric isomers resulted only in decomposition.

(11) (a) Vroman, H. E.; Cohen, C. F. *J. Lipid Res.* **1967**, *8*, 150. (b) de Vries, B. *Chem. Ind. (Britain)* **1962**, 1049. The isomers **3E** and **3Z** were not separable on untreated silica gel under identical conditions. Analysis of isomeric purity was possible by RP hplc (see Experimental Section).

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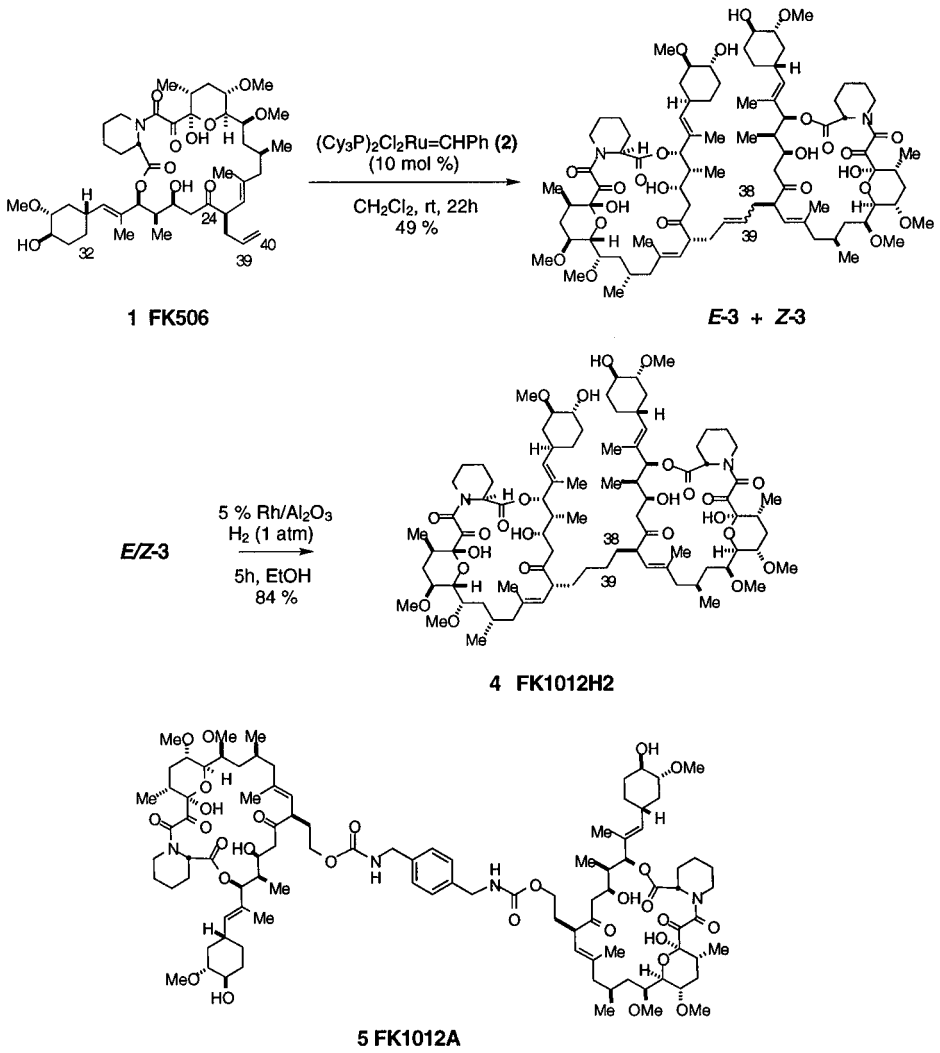
Scheme 1



As an alternative to tedious chromatography, the *E/Z* mixture was hydrogenated (H_2, 1 atm) over 5% Rh/Al_2O_3 for several hours to produce **4**.

This new family of CIDs has a considerably shorter linker than the originally described FK1012s. Therefore, we investigated their ability to bind two proteins simultaneously and to activate cellular pathways by causing cellular proteins to associate with each other. The stoichiometry of binding to FKBP12 was determined by CID-induced inhibition of FKBP12's intrinsic peptidyl-prolyl *cis-trans* isomerase (rotamase) activity.[13] For each of the dimerizers, 1:2 FK1012:FKBP12 binding stoichiometry was observed, and in all cases the first FKBP12-binding event represented by k_i^1 was less than 0.5 nM.[14] At a ligand concentration of *ca*. 58 nM, **4** completely inhibited the rotamase activity of a solution of 116 nM FKBP12. These results are shown in Figure 1 for **3E**, **3Z**, **4**, and **5**. At the inflection point (ca. 58 nM FK1012) **3Z** appears to be the poorest FKBP12 dimerizer ($k_i^2 = 4.8$ nM), and the mixture **3** bound FKBP12 as well as **3Z**.

The new dimerizers **3Z**, **3E**, and **4** were found to be highly effective in functional, cellular assays. A generalized depiction of the CID-induced protein dimerization approach is shown in Figure 2. The discovery that CID-mediated binding events elicit a biological gain-of-function by inducing high effective molarities of proteins forms the basis for the current experiments.[15]

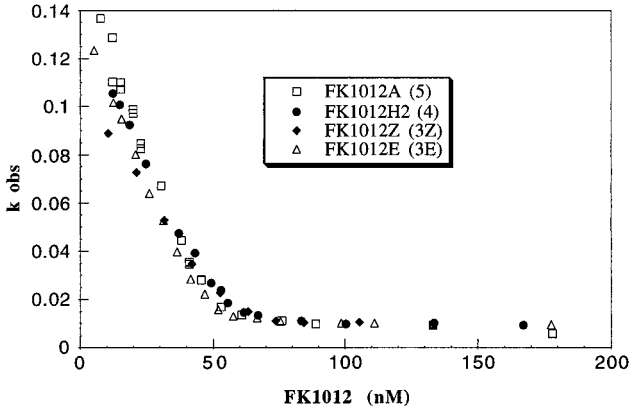


Figure 1. The rotamase activity of 116 nM FKBP12 is inhibited by FK1012s **3E**, **3Z**, **4**, and **5** during the α-chymotrypsin-catalyzed hydrolysis of *N*-succinyl-ALPF-*p*-nitroanilide.

In the first illustration, the CID-induced dimerization of a membrane-anchored fusion protein was used to activate the T-cell signaling pathway. The fusion protein is translated from a DNA construct containing a myristoylation sequence, the

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(14) See Supplemental Material.

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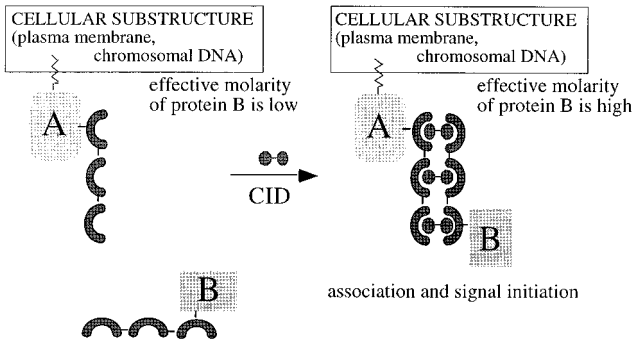

Figure 2. Protein A, fused to three copies of an immunophilin-binding domain (FKBP12, CyP, FRB), is associated with a cellular substructure (plasma membrane, DNA). Adding the appropriate CID (FK1012, $(CsA)_2$, rapamycin) increases the effective molarity of fusion protein B near protein A, leading to signal transduction or transcriptional activation.

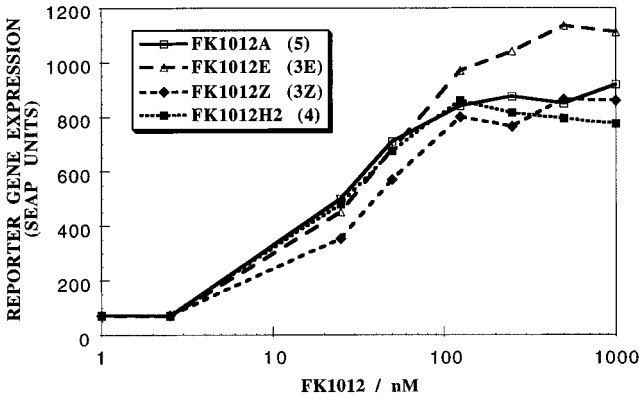

Figure 3. Reporter gene activation by FK1012s **3E**, **3Z**, **4**, and **5** in Jurkat T cells expressing FKBP12 fused to the zeta chain of the T cell receptor. The data were obtained using SEAP as the reporter gene after a 2 h incubation (37 °C) period.

intracellular domain of the T-cell receptor (ζ-chain), and three consecutive FKBP12 dimerization domains. When the protein is dimerized/oligomerized, a secreted alkaline phosphatase (SEAP) reporter gene under the control of the NFAT transcriptional activator is expressed.[1] As shown in Figure 3, each of the CIDs was found to activate the TCR signaling pathway, quantitated by measuring the SEAP enzyme activity. The CIDs **3E**, **3Z**, **4**, and **5** all activate this signaling pathway at similar concentrations, but at higher concentrations, **3E** is somewhat more effective than the other CIDs. At *ca*. 100 nM **3E**, **3Z**, **4**, and **5** registered levels of activation similar to that of the mitogens PMA (50 ng/mL) and ionomycin (1 μM), which are known to activate the TCR pathway.[1]

The new CIDs **3E**, **3Z**, and **4** did not inhibit calcineurin's cellular activity. The well-known immunosuppressive properties of FK506 and cyclosporin A (CsA) emanate from the inhibition of calcineurin's phosphatase activity by FK506-FKBP12 and CsA-cyclophilin A, respectively.[16] Jurkat cells were transiently transfected with cDNA coding for SEAP under the control of the NFAT transcriptional activator.[1] These cells were treated with FK1012s **3E**, **3Z**, and **4** (up to 1 μM) and stimulated with the mitogens PMA and ionomycin. Translocation of NFAT from the cytoplasm to the nucleus is calcineurin-dependent. If the FK1012s **3E**, **3Z**, and **4** inhibited calcineurin following their complexation to cellular FKBP12, they would consequently inhibit the induced SEAP activity. No loss of this activity in the presence of the FK1012s **3E**, **3Z**, and **4** was noted. In contrast, the immunosuppressants FK506 and CsA completely inhibit SEAP gene expression with IC_{50} values of *ca*. 10 nM. From this assay it can also be concluded that none of the dimerizers contain immunosuppressive (monovalent) impurities. The CIDs **3E**, **3Z**, and **4** appear to be nontoxic to mammalian cells even at concentrations considerably above those required to activate their target fusion proteins.

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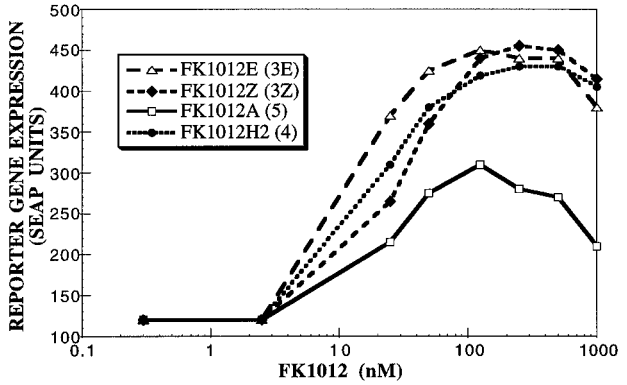

Figure 4. Reporter gene activation by FK1012s **3E**, **3Z**, **4**, and **5** in Jurkat T cells co-expressing FKBP12 fused to a DNA-binding domain and FKBP12 fused to a transcriptional activation domain.[6] The data were obtained using SEAP as the reporter gene after a 2.5 h incubation (37 °C) period.

The performance of the new CIDs was evaluated further in a transcriptional activation experiment.[6] In terms of the generalized dimerization schematic depicted in Figure 2, protein A (the DNA binding domain of Gal4) is bound to DNA. Cytosolic protein B (the transcriptional activation domain of the viral protein vp16) is recruited to the Gal4 fusion protein by the CID, resulting in the creation of a functional transcriptional activator. The increase in effective molarity of the activation domain near the promoter of the SEAP reporter gene results in its enhanced transcription and subsequent translation. The data in Figure 4 demonstrate that the new dimerizers **3E**, **3Z**, and **4** are the most potent of the known homodimeric activators of inducible gene expression. The origin of this enhancement (relative to, e.g., FK1012A) may be due to fewer unproductive intramolecular interactions with the immunophilin domains. Similar enhanced performance has been observed with the CID **4** in the dimerization of a membrane bound Fas cell death domain[17] and in the dimerization of a platelet-derived growth factor receptor.[18]

Conclusion

It has been shown that highly effective CIDs can be synthesized in one step by using olefin metathesis. The olefin metathesis reaction with the Grubbs' ruthenium carbene catalyst has proven to be sufficently mild with these polyfunctional macrolides that no protecting groups need to be used. These new compounds can be used to activate signal transduction and gene expression in cells as well as transgenic animals.[19] The ease of their synthesis will now allow more widespread usage of CIDs, even in animal experiments that typically require larger quantities of CIDs than the cell culture experiments reported here.

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Experimental Section

Rotamase Inhibition Assay. The rotamase assay was conducted at pH 7.9 in 40 mM HEPES ctg 0.1% Triton-X and 5 mM DTT. The peptide substrate used was *N*-succinyl-ALPF-*p*-nitroanilide (Bachem) at 60 mM (prepared fresh and filtered, 0.45 μm) and the FKBP12 concentration was 116 nM (both in HEPES assay buffer). A fresh solution of 50 mg/mL α-Chymotrypsin (Sigma) was prepared in 1 mM HCl(aq) and filtered (0.45 μm). To the solution containing succALPF-pNA and FKBP was added FK1012 inhibitor at a given concentration and the vessel was allowed to stand for 2 h at ambient temperature. The contents were transferred to a cuvette and equilibrated to 4 °C for 12 min, 50 μL of α-chymotrypsin solution (at 4 °C) was added all at once, and data collection (UV absorbance 408−412 nm) was begun. Rate and inhibition constants were extracted with use of the programs PSEUDO and KORE, respectively.[20] Recombinant hFKBP12 was overexpressed as Standaert et al.[21] and analyzed by SDSPAGE and electrospray MS (MW = 11 818 amu). Measured rotamase inhibition values, k_i^1 and k_i^2, were as follows: **3E**, 0.1, 2.1 nM; **3Z**, 0.1, 4.8 nM; **4**, 0.4, 2.1 nM; **3EZ**, 0.1, 3.6 nM.

Melting points are uncorrected. $(Cy_3P)_2Cl_2Ru(=CHPh)$ was commercially obtained from Strem Chemical and handled in a glovebag. Reactions were performed under nitrogen, unless otherwise noted. Dichloromethane was freshly distilled from CaH_2 and passed through activated basic alumina prior to use. Hplc was performed with a ThermoSeparation products system equipped with a 4.6 mm × 150 mm Vydac C-18 column 201HS (Nest Group, Southborough, MA) heated at 70 °C, running a gradient elution from 70/30 0.1% TFA(aq)/CH_3CN to 10/90 0.1% TFA(aq)/CH_3CN over 30 min.

Synthesis of FK1012EZ 3. Into a flame-dried 10-mL schlenk tube equipped with a reflux condensor, magnetic stirbar, and argon inlet was placed 22.8 mg of FK506 **1** (0.0284 mmol, Pfizer) and 2.3 mg of $(Cy_3P)_2Cl_2Ru(=CHPh)$ (**2**, 0.0028 mmol, 0.10 equiv, Strem Chemical) in 0.5 mL of DCM (dist'd from CaH_2 and passed through basic alumina immediately prior to use). This mixture was then stirred under argon for 22 h at room temperature, diluted with DCM (2 mL), and purified by flash chromatography (silica gel, 1.4 × 15 cm) by gradient elution with 12:8:1 DCM/benzene/*i*-PrOH (150 mL) then 9:1:1 DCM/benzene/*i*-PrOH (150 mL) to furnish 11.6 mg (50.8%) of FK506, R_f 0.51 (9:1:1 DCM/benzene/*i*-PrOH), and 11.0 mg (49%) **3**, R_f 0.32, as an off-white solid. Reverse phase hplc (Vydac C-18 column 201HS, 4.6 mm × 150 mm, 70/30 0.1% TFA(aq)/CH_3CN to 10/90 0.1% TFA(aq)/CH_3CN over 30 min at 70 °C) indicated an *ca*. 1:1 mixture of alkene isomers, retention time/min: 28.1 (*Z*), 28.4 (*E*). The isomers were subsequently separated by flash chromatography (Ag-impregnated silica gel, 1.4 × 15 cm) by elution with 9:1:1 DCM/benzene/*i*-PrOH. The *E*-isomer eluted first, followed by mixed fractions, then the *Z*-isomer. **FK1012E (3E):** FAB-MS (NBA, KI) molecular ion calcd (integer masses) for $C_{86}H_{134}N_2O_{24}K$ 1617, found 1617 (M + K); FT-IR (film, cm^{-1}) 3510, 2936, 2826, 1741, 1651, 1450, 1194, 1172; ^1H NMR (500 MHz, $CDCl_3$, ppm) δ 5.34−5.15 (4H, m), 5.08−4.54 (8H, m), 4.39 (2H, d, J = 13.4 Hz), 4.31−4.25 (2H, m), 3.95−3.51 (6H, m), 3.49−3.16 (26H, m), 3.01−2.96 (2H, ddd J = 12.7, 8.6, 4.2 Hz), 2.72−2.62 (4H, m), 2.43−1.22 (56H, m), 1.12−0.53 (24H, m). **FK1012Z (3Z):** FAB-MS (NBA, KI) molecular ion calcd (integer masses) for $C_{86}H_{134}N_2O_{24}K$ 1617, found 1617 (M + K); FT-IR (film, cm^{-1}) 3500, 2934, 1741, 1653, 1482, 1381, 1284, 1196, 1172; ^1H NMR (500 MHz, $CDCl_3$, ppm) δ 5.30−5.14 (4H, m), 5.07−4.57 (8H, m), 4.37 (2H, d, J = 15.1 Hz), 4.30 (2H, m), 3.97−3.54 (6H, m), 3.45−3.11 (26H, m), 2.99 (2H, m), 2.78−2.62 (4H, m), 2.49−1.22 (56H, m), 1.10−0.54 (24H, m).

Synthesis of FK1012H2 4. Into a flame-dried, nitrogen-flushed, 5-mL, round-bottom flask equipped with stirbar and rubber septum was placed 7.9 mg of **3** and 3 mg of 5% Rh/Al_2O_3 in 2 mL of anhydrous EtOH under an atmosphere of H_2 (balloon). The contents were vigorously stirred for 4 h, an additional portion of catalyst (3.0 mg) was added, and stirring was continued for 1 h. Hplc analysis (same conditions as above) indicated complete conversion to a new compound eluting at 28.6 min. The reaction mixture was evaporated (rotary evaporator), diluted with EA (10 mL), filtered (Celite), and purified by chromatography (silica gel, 1.5 × 15 cm) by elution with 9:1:1 DCM/benzene/*i*-PrOH (50 mL) to give 6.5 mg (84%) of **4** as a white solid, mp 125−127 °C, R_f 0.35. FAB-MS molecular ion calcd (integer masses) for $C_{86}H_{136}N_2O_{24}K$ 1619, found 1619 (M + K); FT-IR (film, cm^{-1}) 3500, 2940, 1740, 1705, 1650, 1445, 1101; ^1H NMR (500 MHz, $CDCl_3$, ppm) δ 5.35−5.14 (6H, m), 5.08−4.93 (4H, m), 4.77 (2H, m), 4.58 (2H, m), 4.38 (2H, m), 4.27 (2H, m), 3.94−3.82 (2H, m), 3.65 (2H, m), 3.59−3.47 (2H, m), 3.45−3.16 (24H, m), 3.10 (2H, m), 3.05−2.96 (4H, m), 2.74 (2H, m), 2.64 (2H, m), 2.37−1.11 (52 H, m), 1.10−0.77 (26 H, m). ^{13}C NMR (125 MHz, $CDCl_3$, ppm, data for major isomer) δ 213.4, 196.2, 169.0, 164.7, 138.58, 132.38, 129.68, 129.63, 123.17, 97.03, 96.99, 84.16, 77.88, 75.19, 73.67, 73.63, 73.54, 70.1, 56.95, 56.59, 56.54, 56.29, 52.95, 52.69, 48.60, 43.86, 43.67, 39.81, 39.20, 34.90, 34.87, 34.60, 32.95, 32.70, 31.20, 30.80, 30.63, 27.62, 27.08, 26.20, 24.57, 24.50, 21.20, 20.84, 20.45, 20.42, 16.23, 15.85, 15.68, 14.12, 9.49.

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