Biotinylated N,N'-diarylureas as probes for the activation of adenosine monophosphate-activated kinase (AMPK)

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Abstract: Biotinylated analogs of drug candidates provide useful tools for studying the drug–target protein interactions. Polyhalogenated N,N'diarylureas are potent activators of adenosine monophosphate-activated kinase (AMPK) and potentially promising agents for the treatment of cancer. Various biotinylated versions of these N,N'-diarylureas were synthesized and evaluated in AMPK-activation studies.

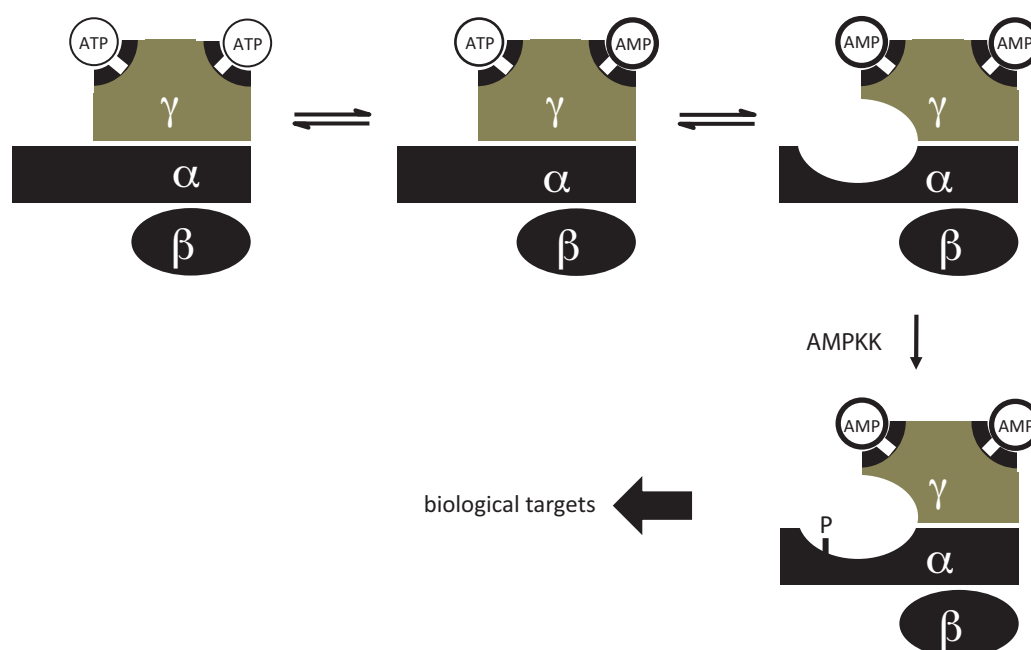**Key words:** Adenosine monophosphate-activated kinase, biotinylated N,N'-diarylureas

1. Introduction

The importance of adenosine monophosphate-activated kinase (AMPK) in regulating energy homeostasis[1−3] gives it a dominant role in cell maintenance and cell cycle progression and makes it an attractive target for new drugs to treat diseases such as obesity, diabetes,[4] and cancer.[5] Activated AMPK plays an essential role in various signaling pathways, important cellular processes that affect lipid, cholesterol, and glucose metabolism. The sequence of events in the activation of the heterotrimeric AMPK involves the initial binding of AMP at the 2 Bateman domains, each composed of 2 cystathione β-synthase subdomains in the γ subunit of AMPK (Figure 1). The AMP-binding event triggers a conformational change in the γ subunit and permits phosphorylation of Thr142 in the associated α subunit. Unraveling the precise location where "small molecule" drug candidates bind AMPK is challenging because of various isoforms for each of the 3 subunits, the binding of various kinases (AMPKK) that phosphorylate AMPK, the binding of allosteric activators, and the numerous interacting partners of the activated AMPK. Any of these stages of activation and interaction are potential targets for therapeutic drugs, and a number of small-molecule activators are now known, including metformin, phenformin, aminoimidazole carboxamide ribonucleotide (AICAR), and A769662, that inhibit or delay tumor progression in animal models.[5]

Understanding the nature of drug–target interactions at a molecular level is a common theme in modern drug discovery and makes use of several approaches including affinity chromatography.[6] Inclusion of a D-(+)-biotin "tag" on drug candidates provides a potential means for defining the loci of drug–protein interactions if

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Dedicated to the memory of Professor Ayhan S. Demir

**Figure 1.** Schematic representation of the activation of adenosine monophosphate-activated kinase (AMPK).

the covalently tethered biotin–drug complex retains biological activity, if the drug exhibits high affinity for the target protein(s), and if the tether between the drug and D-(+)-biotin is sufficient to accommodate binding of streptavidin and the target protein at each of the termini of the tethered biotin–drug complex (Figure 2). Commercially available biotinylation reagents offer a range of tether lengths and chemical handles suitable for attachment to amines, alcohols, thiols, and carboxylate groups.[7] Although biotinylation is a versatile approach for affinity chromatography studies of drug–protein and protein–protein interactions, the best locus for the tethered biotin on a particular drug must be determined on a case-by-case basis. Recently, we reported that N,N'-diarylureas activated AMPK,[8] and we next sought to determine their binding target(s) using biotinylated derivatives.

2. Results and discussion

The addition of 4,5-dichloro-2-nitro, 4,5-difluoro-2-nitro-, 4-trifluoromethyl-2-nitroanilines (**1a–c**) to 4-trifluoromethylthiophenylisocyanate and stannous chloride reduction of the intermediate nitro compounds gave the polyhalogenated N,N'-diarylureas (**2a–c**) (Figure 3). In examining the best sites on the polyhalogenated N,N'-diarylureas where we could position a tethered biotin moiety, we were constrained by the limited number of unsubstituted positions on the aryl rings. We explored 2 sites for the tethered biotin: the C-2 position in the 4,5-dichloro-, 4,5-difluoro-, and 4-trifluoromethylaniline portion of the N,N'-diarylureas (**3–7**) or a carboxamide or sulfonamide group at the C-4 position of the N,N'-diarylureas (**8** and **9**). The goal of these studies was the development of a biologically active N,N'-diarylurea tethered to biotin using an appropriate spacer.

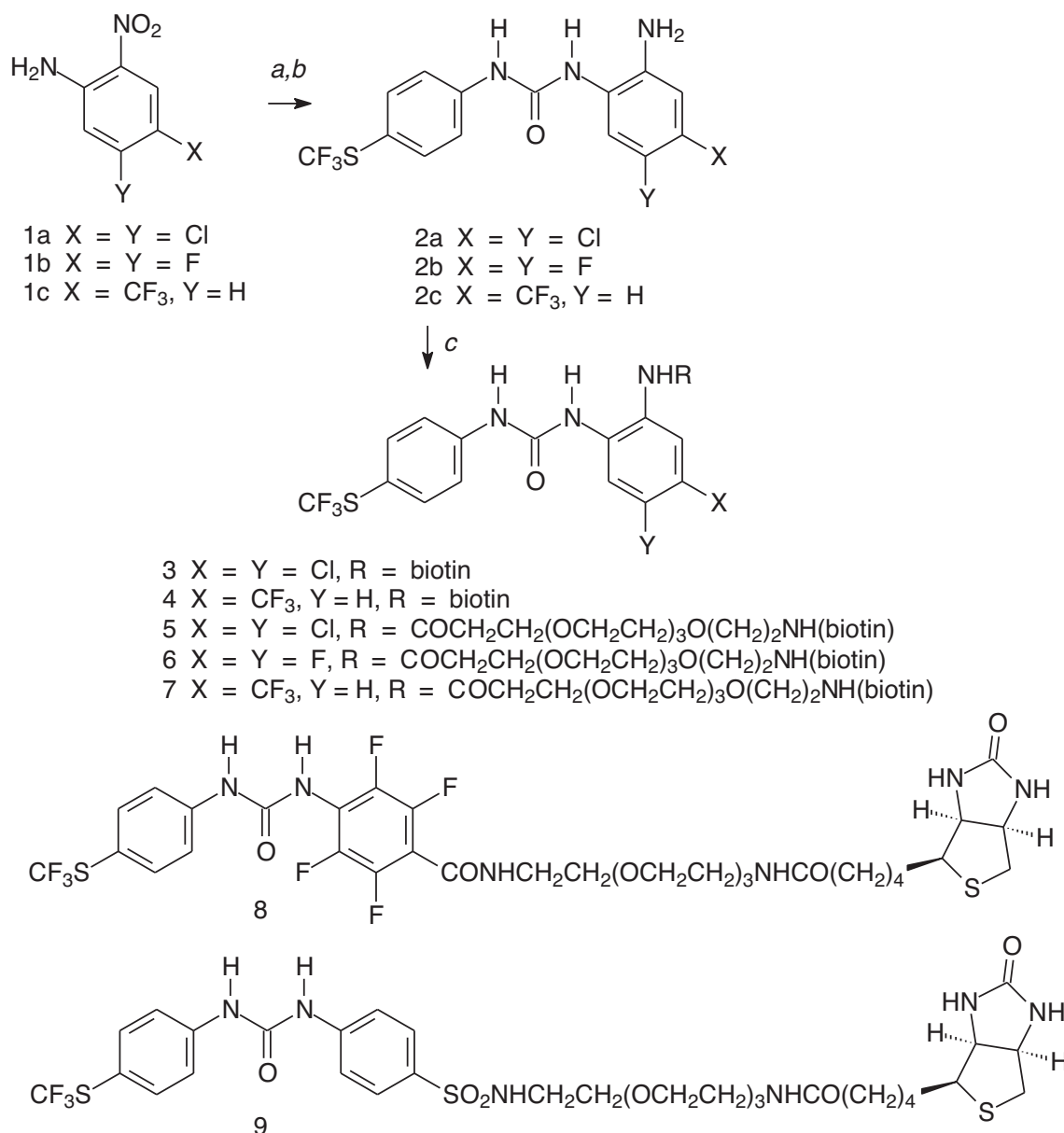### 2.1. Synthesis of biotinylated N,N'-diarylureas

A structure–activity study[8] of N,N'-diarylureas revealed that polyhalogenated N,N'-diarylureas (**2**) were potent AMPK activators if they possessed a 4'-trifluoromethylthio group (4'-CF$_3$S) or a 4'-trifluoromethyl (4'-CF$_3$)
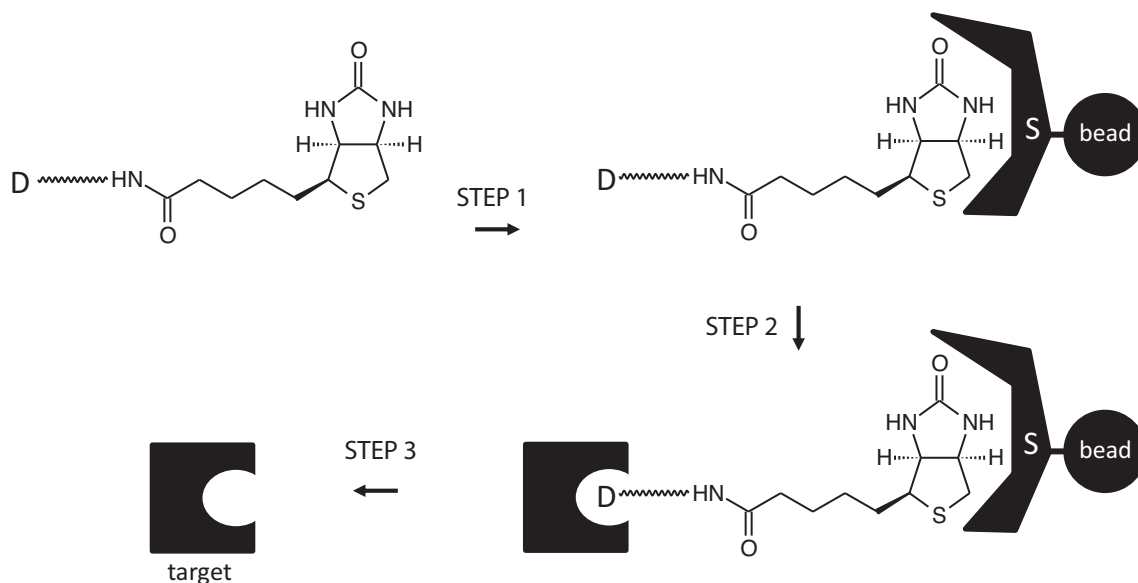
Figure 2. Use of biotinylated N,N'-diarylureas for the identification of their binding site(s) on AMPK. Step 1: binding of tethered biotin-N,N'-diaryl urea to streptavidin beads; Step 2: exposure of beads to cell homogenate (100 plates of LS174T cells) containing potential target(s) and thorough washing of the beads to remove all proteins but the desired target(s); and Step 3: competitive displacement of bound target using 2.5 mM D-(+)-biotin, SDS-PAGE analysis of eluate, and identification of target, including AMPK binding site(s), using mass spectrometry.

group and if they possessed a second aryl ring bearing various chlorine or fluorine substituents. Substitutions with groups other than 4'-trifluoromethylthio or 4'-trifluoromethyl groups or with halogens other than fluorine or chlorine produced inactive compounds. With respect to the second aryl ring, 2 halogenation patterns, either the 4,5-dichloro-substitution in **2a** or the 4,5-difluoro-substitution in **2b**, were most promising in terms of AMPK activation. Consequently, we constructed biotinylated N,N-diarylureas (**3–7**) (Figure 3) that possessed a 4'-trifluoromethylthio group in one aryl ring and one of these polyhalogenation patterns in the other. Other electron-withdrawing groups, such as carboxylic acids or sulfonic acids in the second aryl ring, showed modest AMPK activation and biotinylated carboxamides and sulfonamide versions of N,N-diarylureas (**8** and **9**) that possessed a biotin tethered to one of these groups were also prepared.

The synthesis of these N,N'-diarylureas with and without a polyethylene glycol (PEG) spacer required the introduction of a suitable chemical handle for attaching a tethered D-(+)-biotin to the urea framework. As mentioned earlier, we synthesized the 2-amino-substituted N,N'-diarylureas (**2a–c**). These amino-substituted analogs were coupled to either to D-(+)-biotin or 15-N-(+)-biotinamido-4,7,10,13-tetraoxopentadecanoic acid (d-PEG®-4-biotin acid) to afford the desired, tethered, biotinylated N,N-diarylureas **3–7** (Figure 3). Preparation of an N,N'-diarylurea connected to D-(+)-biotin through a C-4 carboxamide linkage employed the condensation of 4-(trifluoromethylthio)phenylisocyanate with 4-amino-2,3,5,6-tetrafluorobenzoic acid and coupling to (+)-biotinyl-3,6,9-trioxaundecanediamine (amine PEG$_3$-biotin) to afford the biotinylated urea **8**. Finally, in the same fashion, preparation of an N,N'-diarylurea connected to D-(+)-biotin through a C-4 sulfonamide linkage employed the reaction of 4-nitrosulfonyl chloride with amine PEG$_3$-biotin, reduction of the nitro group, and coupling with 4-(trifluoromethylthio)phenylisocyanate to afford the biotinylated urea **9**.

Figure 3. Synthesis of biotinylated N,N'-diarylureas. Legend: a, 4-(trifluoromethylthio)phenylisocyanate; b, SnCl$_2$, HCl; c, either D-(+) biotin, 15-N-(+)-biotinamido-4,7,10,13-tetraoxopentadecanoic acid (d-PEG®-4-biotin acid) or (+)-biotinyl-3,6,9-trioxaundecanediamine (amine-PEG$_3$-biotin), 1-hydroxybenzotriazole hydrate, and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride.

2.2. AMPK activation of biotinylated N,N'-diarylureas

We previously demonstrated that N,N'-diarylureas activated AMPK by analyzing AMPK activation by Western blotting with anti-Phospho-AMPKa (Thr172).[8] The total amount of AMPK was analyzed as a control. We found that 4'-trifluoromethylthiophenyl-3,4-dichlorophenylurea significantly increased phosphorylated AMPK at 1–3 μM concentrations. The biotinylated N,N'-diarylureas **3**–**9** (Figure 3) were evaluated at 10 μM concentrations, but unfortunately they displayed considerably reduced potency relative to nonbiotinylated

counterparts. For example, the biotinylated N,N'-diarylurea **3** showed no activation at 3 μM and only 16% of the AMPK activation at 10 μM that was displayed by its nonbiotinylated N,N'-diarylurea **2a** at 3 μM. As this level of AMPK activation was judged to be too little to proceed, the search for additional biotinylated versions of these promising reagents continues.

3. Experimental

3.1. Chemicals

Chemicals were purchased from Sigma Aldrich, Alfa Aesar, Quanta Biodesign, Ltd. (d-PEG®-4-biotin acid or 15-N-(+)-biotinamido-4,7,10,13-tetraoxopentadecanoic acid), and Thermo Scientific (amine-PEG$_3$-biotin or (+)-biotinyl-3,6,9-trioxaundecanediamine), or the necessary chemicals were synthesized according to literature procedures. N,N'-Diarylureas were prepared as previously described.[8] Solvents were used from commercial vendors without further purification unless otherwise noted. Nuclear magnetic resonance spectra were determined in DMSO-d_6 using a Varian instrument (^{1}H, 400 MHz). The complexity of fluorine–carbon couplings in these polyfluorinated compounds provided little useful structural information and ^{13}C NMR data were not recorded. MALDI mass spectra were obtained on a Bruker Ultraflexstreme time-of-flight mass spectrometer (Billerica, MA, USA), using DHB (2,5-dihydroxybenzoic acid) matrix. Compounds were chromatographed on preparative layer Merck silica gel F254 unless otherwise indicated.

3.2. General procedures for the synthesis of biotinylated ureas

To a suspension of 0.30 mmol (1 equiv) of D-(+) biotin or d-PEG®-4-biotin acid (Quanta Biodesign, Ltd.) in 2 mL of anhydrous DMF was added 48.6 mg (0.36 mmol, 1.2 equiv) of 1-hydroxybenzotriazole hydrate and 69 mg (0.36 mmol, 1.2 equiv) of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride. The mixture was stirred for 10 min and 0.30 mmol (1 equiv) of the appropriate urea was added. The mixture was stirred for 12 h at 25 °C. DMF was evaporated under vacuum, and the products were purified by chromatography.

_N_-(4,5-Dichloro-2-(3-(4-(trifluoromethylthio)phenyl)ureido)phenyl)-5-(2-oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl)pentanamide (3). Yield 25% as white solid (1:5 CH$_3$OH-CH$_2$Cl$_2$, R$_f$ = 0.63). ^{1}H NMR: δ 9.69 (s, 1H, NH), 9.65 (s, 1H, NH), 8.15 (br s, 1H, NH), 8.08 (s, 1H), 7.65–7.60 (m, 5H), 6.43 (s, 1H, NH_biotin_), 6.37 (s, 1H, NH_biotin_), 4.30–4.26 (m, 1H), 4.16–4.11 (m, 1H), 3.12–3.09 (m, 1H), 2.78 (dd, 1H, J$_1$ = 12.2 Hz, J$_2$ = 5.0 Hz), 2.57 (d, 1H, J = 12.4 Hz), 2.40 (t, 2H, J = 7.0 Hz), 1.70–1.33 (m, 6H). MALDI-TOF MS Calcd for C$_{24}$H$_{25}$Cl$_2$F$_3$N$_5$O$_3$S$_2$ [MH+]: 622.0728. Found 622.0733.

5-(2-Oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl)-_N_-(5-(trifluoromethyl)-2-(3-(4-(trifluoromethylthio)phenyl)ureido)phenyl)pentanamide (4). Yield 21% as white solid (1:5 CH$_3$OH-CH$_2$Cl$_2$, R$_f$ = 0.55); ^{1}H NMR: δ 9.73 (s, 1H, NH), 9.71 (s, 1H, NH), 8.26 (br s, 1H, NH), 8.07 (d, 1H, J = 8.8 Hz), 7.66–7.61 (m, 5H), 7.55 (dd, 1H, J$_1$ = 8.8 Hz, J$_2$ = 1.6 Hz), 6.44 (s, 1H, NHbiotin), 6.37 (s, 1H, NHbiotin), 4.32–4.27 (m, 1H), 4.15–4.09 (m, 1H), 3.14–3.09 (m, 1H), 2.78 (dd, 1H, J$_1$ = 12.8 Hz, J$_2$ = 5.2 Hz), 2.57 (d, 1H, J = 12.4 Hz), 2.43 (t, 2H, J = 7.4 Hz), 1.71–1.31 (m, 6H); MALDI-TOF MS Calcd for C$_{25}$H$_{25}$F$_6$N$_5$O$_3$S$_2$Na [MNa+]: 644.1201. Found 644.1191.

_N_-(4,5-Dichloro-2-(3-(4-(trifluoromethylthio)phenyl)ureido)phenyl)-1-(5-(2-oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (5). Yield 34% as white solid (1:5 CH$_3$OH-CH$_2$Cl$_2$, R$_f$ = 0.58). ^{1}H NMR: δ 10.27 (s, 1H, NH), 9.70 (s, 1H, NH), 8.91

(s, 1H, NH), 8.19 (s, 1H), 7.86 (t, 1H, J = 6.0Hz, NH), 7.68–7.62 (m, 5H), 6.42 (s, 1H, NH*biotin*), 6.37 (s, 1H, NH*biotin*), 4.31–4.27 (m, 1H), 4.13–4.09 (m, 1H), 3.72 (t, 2H, J =6.6 Hz), 3.52–3.47 (m, 12H), 3.40–3.35 (m, 2H), 3.19–3.15 (m, 2H), 3.10–3.06 (m, 1H), 2.81 (dd, 1H, J_1 = 12.2 Hz, J_2 = 5.0 Hz), 2.74 (t, 2H, J = 6.8 Hz), 2.57 (d, 1H, J = 12.0 Hz), 2.05 (t, 2H, J = 7.4 Hz), 1.64–1.22 (m, 6H). MALDI-TOF MS Calcd for $C_{35}H_{45}Cl_2F_3N_6O_8S_2Na$ [MNa+]: 891.1967. Found 891.1973.

N-(4,5-Difluoro-2-(3-(4-(trifluoromethylthio)phenyl)ureido)phenyl)-1-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)-3,6,9,12-tetraoxapentadecan-15-amide (6). Yield 35% as white solid (1:5 CH_3OH-CH_2Cl_2, R_f = 0.56). ^{1}H NMR: δ 9.69 (s, 1H, NH), 9.59 (s, 1H, NH), 8.07 (s, 1H, NH), 7.86–7.81 (m, 2H), 7.65–7.60 (m, 4H), 7.44–7.39 (m, 1H), 6.43 (s, 1H, NH*biotin*), 6.37 (s, 1H, NH*biotin*), 4.31–4.28 (m, 1H), 4.13–4.10 (m, 1H), 3.72 (t, 2H, J = 6.2 Hz), 3.52–3.45 (m, 12H), 3.37 (t, 2H, J = 6.0 Hz), 3.19–3.15 (m, 2H), 3.11–3.06 (m, 1H), 2.81 (dd, 1H, J_1 = 12.2 Hz, J_2 = 5.0 Hz), 2.64 (t, 2H, J = 6.2 Hz), 2.57 (d, 1H, J = 12.0 Hz), 2.05 (t, 2H, J = 7.4 Hz), 1.64–1.23 (m, 6H). MALDI-TOF MS Calcd for $C_{35}H_{45}F_5N_6O_8S_2Na$ [MNa+]: 859.2558. Found 859.2561.

1-(5-(2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)-N-(5-(trifluoromethyl)-2-(3-(4-(trifluoromethylthio)phenyl)ureido)phenyl)-3,6,9,12-tetraoxapentadecan-15-amide (7). Yield 18% as white solid (1:5 CH_3OH-CH_2Cl_2, R_f = 0.53). ^{1}H NMR: δ 9.79 (s, 1H, NH), 9.67 (s, 1H, NH), 8.25 (s, 1H, NH), 8.08 (d, 1H, J = 8.0 Hz), 7.83 (t, 1H, J = 5.6 Hz, NH), 7.65–7.61 (m, 5H), 7.55 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.2 Hz), 6.43 (s, 1H, NH*biotin*), 6.37 (s, 1H, NH*biotin*), 4.31–4.28 (m, 1H), 4.13–4.09 (m, 1H), 3.74 (t, 2H, J = 6.6 Hz), 3.55–3.47 (m, 12H), 3.39–3.36 (m, 2H), 3.19–3.14 (m, 2H), 3.10–3.05 (m, 1H) 2.81 (dd, 1H, J_1 = 12.4 Hz, J_2 = 5.2 Hz), 2.68 (t, 2H, J = 6.2 Hz), 2.57 (d, 1H, J = 12.0 Hz), 2.05 (t, 2H, J = 7.2 Hz), 1.64–1.24 (m, 6H). MALDI-TOF MS Calcd for $C_{36}H_{46}F_6N_6O_8S_2Na$ [MNa+]: 891.2620. Found 891.2626.

2,3,5,6-Tetrafluoro-N-(13-oxo-17-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6,9-tri-oxa-12-azaheptadecyl)-4-(3-(4-(trifluoromethylthio)phenyl)ureido)benzamide (8). To a solution of 51 mg (0.12 mmol, 1 equiv) of 2,3,5,6-tetrafluoro-4-(3-(4-(trifluoromethylthio)phenyl)ureido)benzoic acid in 1 mL of anhydrous DMF was added 19.4 mg (0.14 mmol, 1.2 equiv) of 1-hydroxybenzotriazole hydrate and 27.5 mg (0.14 mmol, 1.2 equiv) of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride. The mixture was stirred for 10 min and 50 mg (0.12 mmol, 1 equiv) of amine-PEG$_3$-biotin was added. The mixture was stirred for 12 h at 25 °C. DMF was evaporated under vacuum, and the residue was chromatographed using 1:10 CH_3OH-CH_2Cl_2 (R_f = 0.21) to give 49.0 mg (50%) of **8** as white solid. ^{1}H NMR: δ 9.54 (s, 1H, NH), 9.02 (t, 1H, J = 5.6 Hz, NH), 8.88 (s, 1H, NH), 7.83 (t, 1H, J = 5.6 Hz, NH), 7.65 (d, 2H, J = 9.2 Hz), 7.62 (d, 2H, J = 9.2 Hz), 6.41 (s, 1H, NH*biotin*), 6.35 (s, 1H, NH*biotin*), 4.31–4.28 (m, 1H), 4.14–4.10 (m, 1H), 3.54–3.49 (m, 10H), 3.43 (t, 2H, J = 5.6 Hz), 3.39 (t, 2H, J = 6.0 Hz), 3.20–3.15 (m, 2H), 3.11–3.06 (m, 1H), 2.81 (dd, 1H, J_1 = 12.4 Hz, J_2 = 5.2 Hz), 2.57 (d, 1H, J = 12.0 Hz), 2.06 (t, 2H, J = 7.2 Hz), 1.63–1.23 (m, 6H). MALDI-TOF MS Calcd for $C_{33}H_{40}F_7N_6O_7S_2$ [MH+]: 829.2288. Found 829.2272.

5-(2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(2-(2-(2-(4-(3-(4-(trifluoromethyl-thio)phenyl)ureido)phenylsulfonamido)ethoxy)ethoxy)ethoxy)ethyl)pentanamide (9). A mixture of 100 mg (0.17 mmol) of N-(2-(2-(2-(2-(4-aminophenylsulfonamido)ethoxy)ethoxy)ethoxy)ethyl)-5-(2-oxohexa-hydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide and 31 mg (0.17 mmol) of 4-(trifluoromethylthio)phenyl isocyanate in 2 mL of acetonitrile was stirred at 60 °C for 6 h. After cooling, the reaction mixture was

chromatographed using 1:10 CH_3OH-CH_2Cl_2 ($R_f = 0.3$) to give 59 mg (47%) of **9** as white foam: ^{1}H NMR: δ 9.23 (s, 1H, NH), 9.19 (s, 1H, NH), 7.80 (t, 1H, J = 5.8 Hz, NH), 7.72 (d, 2H, J = 9. 2Hz), 7.66–7.62 (m, 6H), 7.53 (t, 1H, J = 5.8 Hz, NH), 6.40 (s, 1H, NH*biotin*), 6.35 (s, 1H, NH*biotin*), 4.31–4.28 (m, 1H), 4.13–4.10 (m, 1H), 3.49–3.42 (m, 8H), 3.39 (t, 4H, J = 6.0 Hz), 3.19–3.35 (m, 2H), 3.11–3.06 (m, 1H), 2.90–2.85 (m, 2H), 2.81 (dd, 1H, J_1 = 12.4 Hz, J_2 = 5.2 Hz), 2.57 (d, 1H, J = 12.8 Hz), 2.05 (t, 2H, J = 7.4 Hz), 1.63–1.24 (m, 6H). MALDI-TOF MS Calcd for $C_{32}H_{44}F_3N_6O_8S_3$ [MH+]: 793.2329. Found 793.2327.

4. Dedication

This paper is dedicated to the memory of Prof Dr Ayhan S Demir, a postdoctoral fellow at the University of Kentucky in the Watt Laboratory in 1986–1987, a valued colleague and internationally recognized scholar who represented his country, Turkey, and Middle East Technical University well wherever he went.

Acknowledgments

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