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Synthesis of deguelin-biotin conjugates and investigation into deguelin's interactions

José Garcia [a], Sofia Barluenga [a], Katarzyna Gorska [a], Florenz Sasse [b], Nicolas Winssinger [a,*]

[a] Institut de Science et d'Ingénierie Supramoleculaires, Université de Strasbourg, F67000 Strasbourg, France
[b] Department of Chemical Biology, Helmholtz Centre for Infection Research, D-38124 Braunschweig, Germany

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ABSTRACT

Deguelin, a rotenoid, has emerged as an attractive pharmacophore for chemoprevention showing in vivo activity in several xenografts. Recently, several lines of evidence have suggested its mode of action may involve inhibition of HSP90, however binding in a different mode than known pharmacophores. To further probe the target of deguelin and related rotenoids, several biotin conjugates were prepared. None of the conjugates showed significant affinity for HSP90, however two conjugates showed a strong cellular co-localization with mitochondria, consistent with binding to mitochondrial complex 1. Contrarily to rotenone, deguelin and tephrosin were not found to inhibit tubulin polymerization demonstrating a dramatic pharmacological difference between these closely related rotenoids.

1. Introduction

Rotenoids extracted from cubé resin,[1] in particular rotenone (Fig. 1), have long been used as pesticides and insecticides.[2] Closely related analogs (deguelin and tephrosin) have also been shown to be potent cytotoxics. This pharmacophore became the subject of renewed attention following several studies regarding its chemopreventive properties[3,4] showing in vivo activity against breast, colon and skin cancer[5–7] and preventing growth in a mouse xenograft bearing a lung tumor (H1299).[8] It was shown by Pezzuto and coworkers that deguelin most potently inhibited the induction of ornithine decarboxylase[9] which is associated with tumor progression.[10,11] It was later demonstrated that the reduction of ornithine decarboxylase activity in response to rotenoid treatment is a result of NADH/ubiquinone inhibition[12] and that rotenone binds to the PSST subunit of this large multi-protein complex.[13] This inhibition effectively blocks the mitochondrial energetic machinery and aerobic respiration. More recently, Lee and coworkers showed that deguelin selectively promoted apoptosis in non-small-cell lung cancer (NSCLC) cell lines at nM concentration, while having no impact on the cell cycle of primary cells. This effect was correlated to the inhibition of the PI3 K/Akt pathway with a clear reduction of the phosphorylated form of Akt (pAkt) upon treatment with deguelin.[5,14] In addition, the same group showed that HIF-1α was strongly depleted in the presence of deguelin.[8,15] Molecular modeling suggested that deguelin could bind in the nucleotide binding site of Hsp90 in an analogous fashion to 17-AAG, a well characterized inhibitor of Hsp90.[8] However, competition experiments for HSP90 with 17-AAG showed that deguelin and related rotenoids (tephrosin and rotenone) did not compete with 17-AAG for the N-terminal nucleotide binding pocket of HSP90.[16] While deguelin and other rotenoids share some of the pharmacological signature of HSP90 inhibition, treatment of SkBr3 cells with the different rotenoids did not significantly affect the level of HSP90-dependent clients such as ErbB2 known to be highly sensitive to pharmacophores targeting the N-terminal binding site. Similarly, rotenoids failed to promote the degradation of, IP6K2, an HSP90 client sensitive to C-terminal binders.[16] Herein we report further investigations into the target(s) of deguelin and related rotenoids, the synthesis of rotenoid-biotin conjugates and their localization within cells.

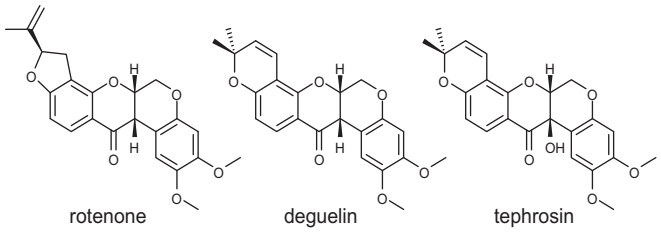

Figure 1. Structure of selected rotenoids.

* Corresponding author. Tel.: +33 368 855 113.
E-mail address: winssinger@isis.u-strasbg.fr (N. Winssinger).

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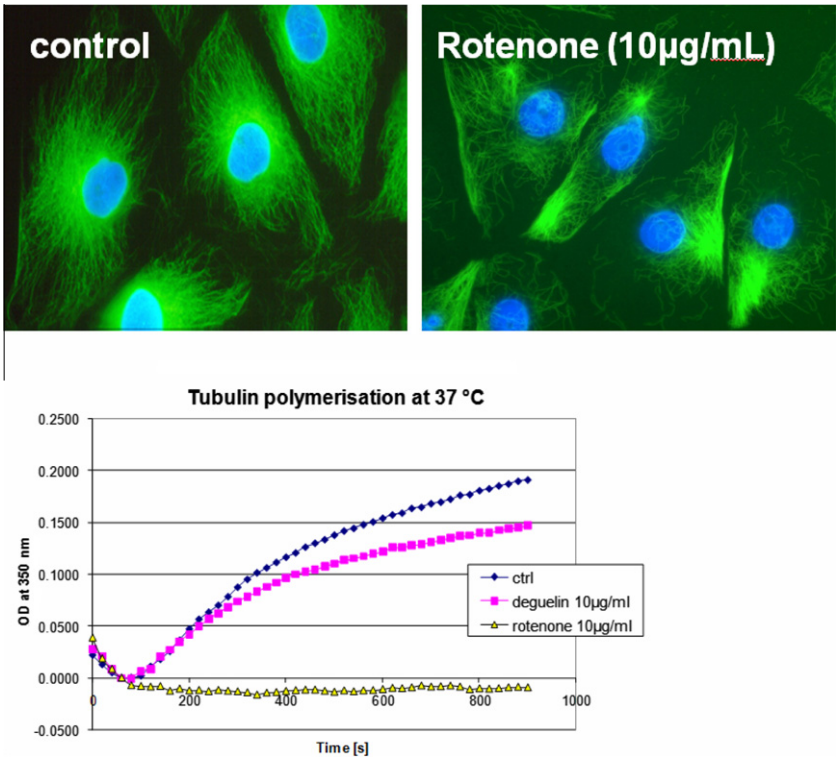
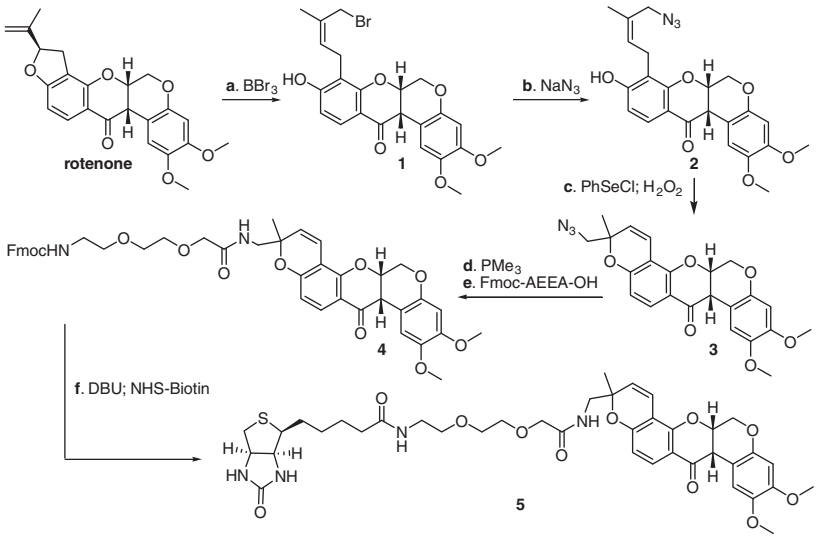


Figure 2. Inhibition of tubulin polymerization by rotenoids. Top: Control cells (left) and rotenone (10 μg/mL) treated cells (right); false coloring: blue for nuclei and chromosomes stained with DAPI; green for tubulin stained with a specific antibody against α-tubulin. Bottom: Tubulin polymerization assay (blue: control; pink: deguelin (10 μg/mL); yellow: rotenone (10 μg/mL)).

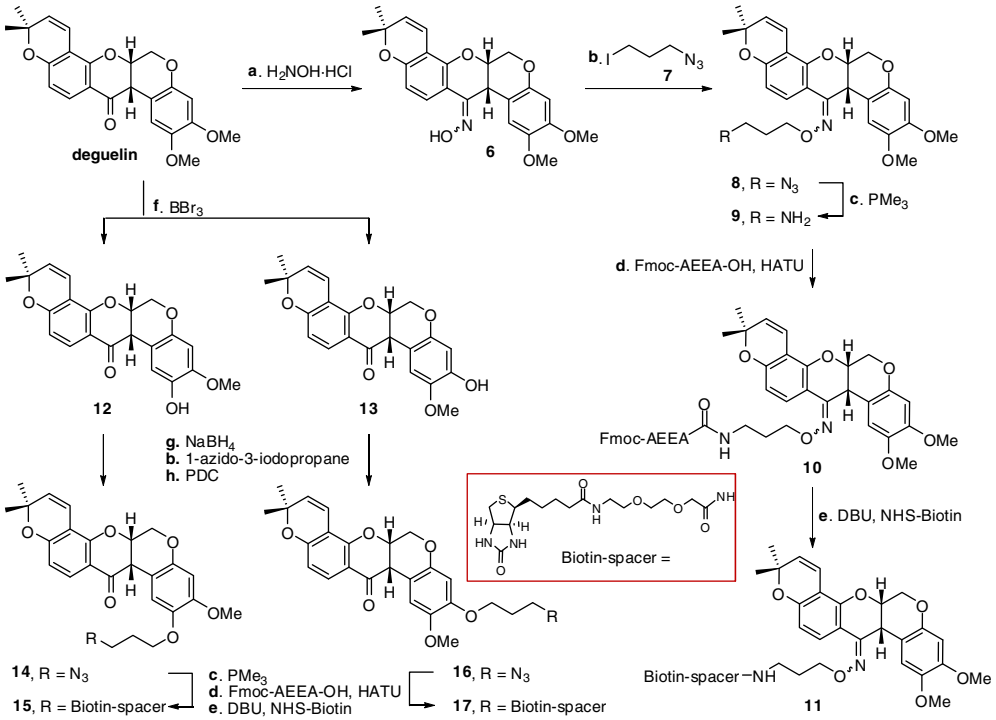
2. Results and discussion

It was reported in the 70's that rotenone inhibits tubulin self-assembly in an analogous fashion to vinca alkaloids and colchicines.[17] As a first step, we asked whether deguelin or tephrosin also had an impact on tubulin dynamics. As shown in Figure 2, treatment of PtK2 cells with 10 μg/ml of rotenone (25 μM) showed a strong disruption of tubulin assembly in a cellular context as well as in an in vitro tubulin assembly assay concurring the fact that the tubulin inhibition is the result of a direct interaction between rotenone and tubulin. However, neither deguelin nor tephrosin (data not showed) showed similar effects suggesting the benzofuran portion of the pharmacophore is essential for the interaction with tubulin. We next investigated if we could validate an interaction between HSP90 and deguelin using biotin conjugates. We envisioned appending the biotin at multiple sites of the molecule to minimize the possibility of perturbing its endogenous interactions. Furthermore, we planned to incorporate a 7.5 Å polyethylene glycol spacer (PEG) to preclude steric hindrance. Four positions were identified for modifications: the pyran moiety, the ketone and either phenolic methyl ether. To append the substituent off the benzopyran moiety, it was anticipated that starting from readily available rotenone would be most convenient. As shown in Scheme 1, treatment of rotenone with BBr_3 afforded known allylic bromide **1**[18] which was treated with NaN_3 to obtain compound **2**. Phenyl selenium chloride mediated cyclization followed by oxidative elimination afforded azide-functionalized benzopyran **3** as a diastereomeric mixture (1:1). Without *a priori* indications regarding a preferred stereochemistry, no efforts to resolve this mixture were made. The azide was used to append the spacer and biotin in a four step sequence which proved practical and robust. Reduction of the azide followed by coupling to a short Fmoc-protected spacer (AEEA: 2-aminoethoxyethoxyacetic acid) afforded intermediate **4**. The Fmoc was removed under the action of DBU and rather than isolating this highly water soluble compound, the crude mixture was treated with HOBt and *N*-hydroxy succinimide activated biotin to afford the first conjugate **5**. In order to conjugate the biotin to the ketone moiety, deguelin was treated with hydroxylamine (Scheme 2) to obtain oxime **6** as a mixture of *E*:*Z* isomers (1:1). In the absence of information suggesting which isomer would be preferable, the oximes were carried forward as a mixture. It is noteworthy that modifications in this area of the molecule do not abrogate its biological activity[19] however, oxime derivatives had thus far not been prepared. The oxime was substituted with 1-azido-3-iodopropane[20] thus affording **8** which was converted to the biotinylated analog **11** following the same four step sequence as for **5**. In order to append the biotin *in lieu* of the phenolic methyl ether, deguelin was treated with BBr_3 which afforded a separable mixture of deprotected phenols. Direct alkylation of the phenols proved unproductive due to the acidity of the benzylic proton α to the carbonyl. However, a temporary reduction of this carbonyl allowed for smooth alkylation of either phenol to obtain intermediates **14** and **16** after reoxidation of the benzylic alcohol (PDC). Using the same sequence of reactions as for **5**, the biotin conjugates **15** and **17** were obtained from **14** and **16**, respectively.

With the biotin conjugates in hand, we investigated their binding to HSP90. The compounds **5**, **11**, **15** and **17** were immobilized on a streptavidin resin and exposed to purified human HSP90 or SkBr3 crude cell lystates (cytosolic fraction). In none of the cases binding of HSP90 could be detected. We next asked whether we could obtain relevant information regarding the target of deguelin from its cellular localization. To this end, solutions containing an excess of compounds **5**, **11**, **15** and **17** (0.5 mM) were individually incubated with a solution of fluorescein-labeled streptavidin (1 mg/mL, 20 μM) in order to saturate all binding sites of streptavidin. After 1 h incubation at 4 °C, the excess of biotin conjugate



Scheme 1. Reagents and conditions: (a) BBr_3 (1.0 equiv), CH_2Cl_2, −10 °C, 30 min, 50%; (b) NaN_3 (1.0 equiv), DMSO, 23 °C, 3 h; (c) PhSeCl (1.0 equiv), H_2O_2 (6.0 equiv), CH_2Cl_2, −30 °C, 1 h, 30% (two steps); (d) PMe_3 (2.0 equiv), 9/1 THF/H_2O, 23 °C, 2 h; (e) DIPEA (1.1 equiv), Fmoc-AEEA-OH (1.1 equiv), HATU (1.1 equiv), DMF, 23 °C, 15 min, 74% (two steps); (f) DBU (1.0 equiv), HOBt (1.5 equiv), biotin *N*-hydroxysuccinimide ester (1.0 equiv), 23 °C, 15 min, 36%. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; DIPEA = diisopropylethylamine; DMF = dimethylformamide; DMSO = dimethylsulfoxide; Fmoc = fluorenylmethyloxycarbonyl; Fmoc-AEEA-OH = [2-(2-(Fmoc-amino)ethoxy)ethoxy]acetic acid; HATU = *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate; HOBt = 1-hydroxybenzotriazole; NHS-Biotin = (+)-biotin *N*-hydroxysuccinimide ester, THF = tetrahydrofuran.



Scheme 2. Reagents and conditions: (a) $H_2NOH{\cdot}HCl$ (10 equiv), NaOH (10 equiv), H_2O (0.03 M), EtOH, 60 °C, 2 h, 67%; (b) 1-azido-3-iodopropane (1.5 equiv), Cs_2CO_3 (1.5 equiv), DMF, 23 °C, 30 min; (c) PMe_3 (2.0 equiv), 9/1 THF/H_2O, 23 °C, 2 h; (d) DIPEA (1.1 equiv), Fmoc-AEEA-OH (1.1 equiv), HATU (1.1 equiv), DMF, 23 °C, 15 min; (e) DBU (1.0 equiv), HOBt (1.5 equiv), biotin *N*-hydroxysuccinimide ester (1.0 equiv), 23 °C, 15 min; (f) BBr_3 (1.0 equiv), CH_2Cl_2, −10 °C, 15 min, 75%; (g) $NaBH_4$ (4.0 equiv), MeOH, 0 °C, 15 min; (h) PDC (1.5 equiv), 4 Å molecular sieves, CH_2Cl_2, 23 °C. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; DIPEA = diisopropylethylamine; DMF = dimethylformamide; Fmoc-AEEA-OH = [2-(2-(Fmoc-amino)ethoxy)ethoxy]acetic acid; HATU = *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate; HOBt = 1-hydroxybenzotriazole; NHS-Biotin = (+)-biotin *N*-hydroxysuccinimide ester; PDC = pyridinium dichromate; THF = tetrahydrofuran.

was removed by size exclusion filtration (30 kDa spin filter). The fluorescent adducts were then added to fixed and permeabilized MCF7 cells. In parallel, the mitochondria were stained with Mito-Tracker Red (Molecular Probes) and the nuclei were stained with DAPI. As shown in Figure 3, treatment with adducts of compound **15** (panel D) and **17** (panel E) gave significantly higher fluorescence signal than treatment with adducts **5** and **11** or FITC-streptavidin alone (panel B, C, F, respectively). This suggests that derivatization of the pyran moiety or formation of oximes attenuate binding relatively to modification of either phenolic methyl ether. Comparing

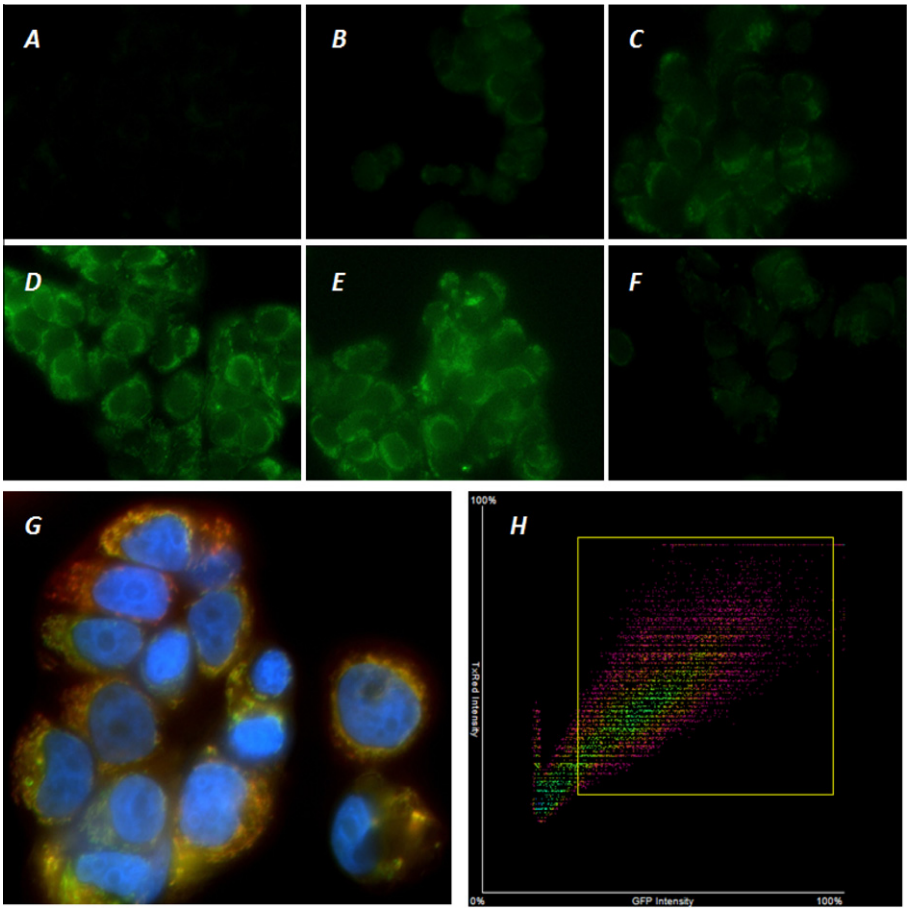


Figure 3. Cellular localization of deguelin-biotin conjugates. A–F: MCF7 cells were fixed, treated with FITC-labeled streptavidin adduct of compounds **5**, **11**, **15**, **17**: untreated cells - negative control (A), compound **5** (B), **11** (C), **15** (D), **17** (E), streptavidin-FITC alone (F); G: superposition of compound **15** (green channel), mitochondria (red channel) and nucleus (blue channel); H: 2-D co-localization fluorogram generated from the green channel (*x*-axis) and red channel (*y*-axis).

the localization of deguelin conjugate to mitochondrial gave a strong overlap for both compounds (see panel G for the overlap of channels corresponding to compound **15**, mitochondria and nucleus). Analysis of the co-localization by 2-D plot indeed shows a high correlation giving a Mander's overlap of 0.97.[21,22] The strong co-localization with mitochondria observed with conjugates **15** and **16** are consistent with NADH/ubiquinone oxidoreductase (complex 1) binding but not with HSP90 binding.

3. Conclusion

In conclusion, the small structural differences between rotenone and deguelin (furan vs pyran) do contribute to an important pharmacological difference in their aptitude to inhibit tubulin polymerization. Taken together, the presented data do not show any evidence of interaction between deguelin and HSP90. While we cannot rule out that the modifications used in the conjugation preclude binding to HSP90 or one of its cochaperones, the data do support deguelin's interaction with mitochondrial complex 1. The inhibition of the Akt pathway previously observed in response to deguelin treatment in oncogenic cell lines may be a downstream product of mitochondrial inhibition. Interestingly, rotenone, which also inhibits mitochondrial complex I at lower concentration than it perturbs tubulin dynamics was identified in a screen for small molecules suppressors of cell death in neuronal cell culture model of Huntington's disease.[23] In this context, rotenone activated the Erk and Akt pathways. The specific staining of mitochondrial complex 1 with the biotin-deguelin conjugates may find application for cellular imaging and does provide important structure activity information.

4. Experimental

4.1. Chemistry

4.1.1. General techniques

All reactions were carried out under a nitrogen atmosphere with dry solvents, unless otherwise noted. Anhydrous solvents were obtained by passing them through commercially available alumina columns (Innovative technology, Inc., MA). Reactions were monitored by LC–MS or thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as visualizing agent and 10% ethanolic phosphomolybdic acid or vanillin solution and heat as developing agents. E. Merck silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography. NMR spectra were recorded on Bruker Advance-400 instrument at 400 (^{1}H) and 100 (^{13}C) MHz. Chemical shifts are given in parts per million (δ) and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: app = apparent, b = broad, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, m = multiplet, q = quadruplet, quint = quintuplet, s = singlet. LC–MS were recorded using an Agilent 1100 HPLC and Surveyor MSQ spectrometer (Thermo Scientific). Unless otherwise stated, Supelco C18 (5 cm $\times$ 4.6 mm, 5 mm particles) column was used with a linear elution gradient from 95% H_2O

(0.5% HCO_2H) to 100% MeCN in 8 min at a flow rate of 0.5 mL/min. The MALDI spectra were measured using a Brucker Daltonics Autoflex TOF spectrometer.

4.1.2. General procedures

4.1.2.1. General procedure for the reduction of azides. Trimethylphosphine (2.0 equiv) was added to a stirring solution of the corresponding azide (1.0 equiv) in 9:1 mixture of THF/H_2O (0.1 M) at 23 °C. After stirring for 2 h at this temperature, the reaction was quenched by adding water and was extracted with EtOAc (×3). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was used in the next step without further purification.

4.1.2.2. General procedure for the coupling of amines with Fmoc-AEEA-OH spacer. Diisopropylethylamine (1.1 equiv) was added to a solution containing Fmoc-AEEA-OH (1.1 equiv) and HATU (1.1 equiv) in DMF (0.5 M) at 23 °C. After stirring for 15 min, a solution of the corresponding amine in DMF (0.5 M) was added and the mixture was stirred for additional 15 min. The reaction was quenched with water and extracted with EtOAc (×3). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography as indicated for each particular example.

4.1.2.3. General procedure for the coupling of amines with biotin *N*-hydroxysuccinimide ester. DBU (1.0 equiv) was added to a solution of Fmoc-protected amine (1.0 equiv) in DMF (0.5 M) at 0 °C. After stirring for 10 min at this temperature, HOBt (1.5 equiv) and biotin *N*-hydroxysuccinimide ester (1.1 equiv) were subsequently added and the reaction was stirred for additional 15 min. The solvent was then removed under reduced pressure and the residue was purified by column chromatography as indicated for each particular example.

4.1.3. Specific procedures and analytical data

4.1.3.1. Bromide 1. BBr_3 (1.0 equiv, 1.27 mL, 1.0 M in hexanes) was added drop-wise to a stirring solution of rotenone (1.0 equiv, 500 mg, 1.27 mmol) in CH_2Cl_2 (1.5 mL) at −10 °C. After stirring for 30 min at this temperature, the reaction was quenched with water (1.0 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 65/35 petroleum ether/EtOAc) to afford bromide **1** (300 mg, 50%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.70 (d, J = 8.7 Hz, 1H), 6.75 (s, 1H), 6.52 (d, J = 8.7 Hz, 1H), 6.44 (s, 1H), 5.62 (dd, J = 7.0, 7.0 Hz, 1H), 4.91 (dd, J = 3.0, 3.0 Hz, 1H), 4.62 (dd, J = 12.0, 3.0 Hz, 1H), 4.18 (d, J = 12.0 Hz, 1H), 3.91 (s, 2H), 3.83 (d, J = 3.9 Hz, 1H), 3.78 (s, 3H), 3.72 (s, 3H), 3.37 (t, J = 7.0 Hz, 2H), 1.91 (s, 3H) (OH signal is not visible) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 23 °C): δ 190.4, 161.6, 160.5, 149.4, 147.6, 145.6, 132.7, 128.0, 127.2, 113.9, 112.6, 110.5 (×2), 104.6, 100.9, 72.1 66.2, 56.3, 55.8, 44.2, 41.6, 22.3, 14.7 ppm. LC–MS (t_R = 1.80 min) m/z $[M+H]^+$ calcd for $C_{23}H_{23}{}^{79}BrO_6$ and $C_{23}H_{23}{}^{81}BrO_6$ 475.07 and 477.07; found: 474.99 and 476.98.

4.1.3.2. Azide 2. Sodium azide (1.0 equiv, 82 mg, 1.26 mmol) was added to a solution of bromide **1** (1.0 equiv, 600 mg, 1.26 mmol) in DMSO (1.5 mL) at 23 °C. After the solution was stirred for 3 h, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 65/35 petroleum ether/EtOAc), to afford 470 mg of desired azide **2**, which was used in the next step without further purification.

4.1.3.3. Azide 3. Phenylselenyl chloride (1.0 equiv, 210 mg, 1.1 mmol) was added to a solution of azidophenol **2** (1.0 equiv, 470 mg, 1.1 mmol) in CH_2Cl_2 (1.5 mL) at −30 °C. The reaction was allowed to reach 23 °C over a period of 1 h and the solvent was removed under reduced pressure. The residue was then dissolved in THF (5.0 mL) and H_2O_2 (6.0 equiv, 6.0 mL, 6.6 mmol) was added at 0 °C. After stirring for 2 h at 23 °C, water (2.0 mL) was added and the solution was extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, 70/30 petroleum ether/EtOAc) to afford azide **3** (170 mg, 30% over two steps) as an oil as 1:1 mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.79 (d, J = 8.7 Hz, 1H), 7.78 (d, J = 8.7 Hz, 1H), 6.83 (d, J = 10.1 Hz, 1H), 6.82 (d, J = 10.1 Hz, 1H), 6.77 (s, 2H), 6.54 (d, J = 8.7 Hz, 1H), 6.52 (d, J = 8.7 Hz, 1H), 6.46 (s, 1H), 6.45 (s, 1H), 5.49 (d, J = 10.1 Hz, 2H), 4.95–4.92 (m, 2H), 4.64 (dd, J = 12.2, 2.6 Hz, 2H), 4.20 (d, J = 12.2 Hz, 2H), 3.86 (d, J = 3.4 Hz, 2H), 3.81 (s, 6H), 3.77 (s, 6H), 3.46–3.29 (m, 4H), 1.51 (s, 3H), 1.42 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 23 °C): δ 189.0 (×2), 159.0 (×2), 156.8 (×2), 149.4 (×2), 147.3 (×2), 143.8 (×2), 129.1, 129.0, 124.3, 124.2, 118.5, 118.3, 113.2 (×2), 111.1, 111.0, 110.3 (×2), 108.5 (×2), 104.5, 104.4, 100.8 (×2), 79.9, 79.6, 72.4 (×2), 66.1 (×2), 58.5 (×2), 56.2 (×2), 55.7 (×2), 44.3 (×2), 24.4, 24.3 ppm; LC–MS (t_R = 1.94 min) m/z $[M]^+$ calcd for $C_{23}H_{21}N_3O_6$ 435.14; found: 435.73.

4.1.3.4. Amide 4. Following the general procedure described for the reduction of azides, azide **3** (1.0 equiv, 33 mg, 76 μmol) gave corresponding amine (structure not shown) as a yellow oil and as 1:1 mixture of diastereomers, which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.76 (d, J = 8.7 Hz, 1H), 7.75 (d, J = 8.7 Hz, 1H), 6.82–6.76 (m, 4H), 6.50–6.44 (m, 2H), 6.46 (s, 2H), 5.49 (d, J = 10.1 Hz, 1H), 5.47 (d, J = 10.1 Hz, 1H), 4.92–4.91 (m, 2H), 4.66–4.62 (m, 2H), 4.18 (d, J = 12.0 Hz, 2H), 3.86 (d, J = 3.4 Hz, 2H), 3.81 (s, 6H), 3.77 (s, 6H), 2.89–2.78 (m, 4H), 1.38 (s, 3H), 1.31 (s, 3H) (NH_2 signal is not visible) ppm. LC–MS (t_R = 1.28 min) m/z $[M]^+$ calcd for $C_{23}H_{23}NO_6$ 409.15; found: 409.20.

According to the general procedure described for the coupling of amines with Fmoc-AEEA-OH spacer, above amine (1.0 equiv, 33 mg, 81 μmol) provided, after purification by column chromatography (SiO_2, 95/5 CH_2Cl_2/MeOH), amide **4** (44 mg, 74% over two steps) as a yellow oil and as 1:1 mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.77–7.74 (m, 6H), 7.58 (d, J = 7.3 Hz, 4H), 7.37 (dd, J = 7.2, 7.2 Hz, 4H), 7.29 (d, J = 7.2 Hz, 4H), 7.10 (b, 1H), 6.77–6.70 (m, 4H), 6.46–6.43 (m, 4H), 5.56 (d, J = 10.4 Hz, 1H), 5.51 (d, J = 10.4 Hz, 1H), 4.87 (b, 2H), 4.60 (dd, J = 12.0, 2.9 Hz, 2H), 4.43 (dd, J = 13.2, 6.6 Hz, 4H), 4.21 (d, J = 6.0 Hz, 2H), 4.16 (d, J = 12.0 Hz, 2H), 3.99 (s, 2H), 3.83 (b, 3H), 3.80 (s, 3H), 3.78 (s, 3H), 3.76 (s, 3H), 3.74 (s, 3H), 3.60–3.48 (m 8H), 3.43–3.32 (m, 8H), 3.22–3.16 (m, 4H), 1.41 (s, 3H), 1.32 (s, 3H) (4 NH signals are not visible) ppm. LC–MS (t_R = 1.98 min) m/z $[M]^+$ calcd for $C_{44}H_{44}N_2O_{11}$ 776.29; found: 776.62.

4.1.3.5. Biotin conjugate 5. In accordance with the general procedure described for the coupling of amines with biotin *N*-hydroxysuccinimide ester, protected amine **4** (1.0 equiv, 22 mg, 28 μmol) gave, after purification by HPLC (Zorbax C18, 9.4 cm × 25 mm, 5 μm particle size, gradient from 5% CH_3CN in 95% H_2O and 0.01% TFA to 95% CH_3CN in 5% H_2O with 0.01% TFA, 3 mL/min, over 45 min, t_R = 19.6, 19.8 min), biotinylated amide **5**

(8.0 mg, 36%) as a brown solid and as 1:1 mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.76 (d, J = 8.7 Hz, 2H), 6.76 (d, J = 8.7 Hz, 4H), 6.46 (d, J = 10.1 Hz, 2H), 5.58 (d, J = 10.0 Hz, 1H), 5.56 (d, J = 10.0 Hz, 1H), 4.94 (d, J = 4.9 Hz, 2H), 4.64 (dd, J = 12.2, 2.8 Hz, 2H), 4.54 (b, 2H), 4.36 (b, 4H), 4.20 (d, J = 12.0 Hz, 1H), 4.18 (d, J = 12.0 Hz, 1H), 4.01 (s, 2H), 3.84 (dd, J = 12.9, 4.4 Hz, 2H), 3.83 (s, 2H), 3.81 (s, 6H), 3.77 (s, 3H), 3.76 (s, 3H), 3.62 (b, 4H), 3.55 (b, 4H), 3.49 (b, 4H), 3.43–3.82 (m, 4H), 3.24–3.15 (m, 6H), 2.95–2.90 (m, 2H), 2.73 (d, J = 12.9 Hz, 2H), 2.20 (t, J = 6.8 Hz, 4H), 1.70–1.29 (m, 8H), 1.36 (s, 3H), 1.30 (s, 3H), 1.29 (m, 4H) (4 NH signals are not visible) ppm; LC–MS (t_R = 1.42 min) m/z $[M]^+$ calcd for $C_{39}H_{48}N_4O_{11}S$ 780.30; found: 780.01. HRMS (MALDI) m/z $[M+H]^+$ calcd for $C_{39}H_{49}N_4O_{11}S$: 781.3113; found: 781.3123.

4.1.3.6. Oxime 6. A solution containing deguelin (1.0 equiv, 15 mg, 38 μmol), hydroxylamine hydrochloride (10 equiv, 26 mg, 0.4 mmol), sodium hydroxide (10 equiv, 15 mg, 0.4 mmol) and H_2O (15 μL) in EtOH (1 mL) was heated at 60 °C for 2 h. The reaction was then neutralized by adding 10% aqueous HCl solution (1.0 mL) and water (1.0 mL). The aqueous layer was extracted with EtOAc (3 × 2.0 mL) and the combined organic layers were dried over Na_2SO_4 and filtered. The solvent was concentrated under reduced pressure and the residue was purified by column chromatography (SiO_2, from 70/30 petroleum ether/EtOAc to 100% EtOAc) to afford oxime **6** (10 mg, 67%) as a foam. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 9.82 (s, OH), 7.52 (d, J = 8.6 Hz, 1H), 6.70 (s, 1H), 6.70 (d, J = 10.0 Hz, 1H), 6.47 (d, J = 8.6 Hz, 1H), 6.44 (s, 1H) 5.58 (d, J = 10.0 Hz, 1H), 4.98 (d, J = 9.0 Hz, 1H), 4.78 (d, J = 9.0, 1H), 4.59 (d, J = 12.0 Hz, 1H), 4.07 (d, J = 12.0 Hz, 1H), 3.77 (s, 3H), 3.60 (s, 3H), 1.43 (s, 3H), 1.41 (s, 3H) ppm; ^{13}C NMR (100 MHz, acetone-d_6, 23 °C): δ 163.7, 157.5, 155.6, 151.7, 151.5, 146.0, 132.0, 131.0, 117.9, 114.8, 111.5, 111.4, 109.8, 108.8, 103.9, 81.5, 78.4, 65.9, 57.5, 57.0, 46.7, 29.2, 29.0 ppm.

4.1.3.7. Azide 8. A solution containing oxime **6** (1.0 equiv, 43 mg, 0.10 mmol), 1-azido-3-iodopropane (1.5 equiv, 34 mg, 0.10 mmol) and Cs_2CO_3 (1.5 equiv, 51 mg, 0.10 mmol) in DMF (1.0 mL) was stirred for 30 min at 23 °C. The reaction was quenched with water (1.0 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 75/25 petroleum ether/EtOAc) to yield azide **8** (35 mg, 70%) as an oil. ^{1}H NMR (400 MHz, acetone-d_6, 23 °C): δ 7.09 (d, J = 8.4 Hz, 1H), 6.71 (d, J = 10.0 Hz, 1H), 6.55 (d, J = 8.4 Hz, 1H), 6.46 (s, 1H), 6.43 (s, 1H), 5.84 (d, J = 10.0 Hz, 1H), 5.22 (ddd, J = 10.8, 2.2, 2.2 Hz, 1H), 5.08 (d, J = 10.8 Hz, 1H), 4.34 (dd, J = 12.4, 2.3, 1H), 3.96 (d, J = 12.4, 2.3 Hz, 1H), 3.95–3.87 (m, 2H), 3.70 (s, 3H), 3.63–3.59 (m, 2H), 3.41 (s, 3H), 2.12–2.06 (m, 2H), 1.42 (s, 3H), 1.41 (s, 3H) ppm; ^{13}C NMR (100 MHz, acetone-d_6, 23 °C): δ 159.6, 157.4, 155.2, 151.7, 151.2, 146.0, 133.2, 132.6, 118.1, 117.7, 117.2, 114.5, 113.9, 112.4, 103.6, 81.4, 78.1, 74.1, 67.7, 57.1, 56.9, 49.9, 48.9, 31.0, 29.0, 28.9 ppm; LC–MS (t_R = 2.05 min) m/z $[M]^+$ calcd for $C_{26}H_{28}N_4O_6$: 492.20; found: 492.07.

4.1.3.8. Amine 9. Following the general procedure described for the reduction of azides, azide **8** (1.0 equiv, 33 mg, 80 μmol) gave amine **9** as a yellow oil and as *E/Z* mixture of oximes, which were then used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.01 (d, J = 8.4 Hz, 1H), 6.98 (d, J = 8.4 Hz, 1H), 6.60 (d, J = 10.0 Hz, 1H), 6.59 (d, J = 10.0 Hz, 1H), 6.52 (d, J = 8.4 Hz, 2H), 6.43 (s, 2H), 6.39 (s, 1H), 6.37 (s, 1H), 5.67 (d, J = 10.0 Hz, 2H), 5.19–5.14 (m, 2H), 4.96 (d, J = 10.8 Hz, 2H), 4.40–4.35 (m, 2H), 3.98 (dd, J = 12.3, 2.1 Hz, 2H), 3.91–3.84 (m, 4H), 3.81–3.73 (m, 4H), 3.77 (s, 6H), 3.47 (s, 6H), 1.93–1.87 (m, 4H), 1.42 (s, 6H), 1.41 (s, 6H) (2 NH_2 signals are not visible) ppm; LC–MS (t_R = 1.47 min) m/z $[M]^+$ calcd for $C_{26}H_{30}N_2O_6$: 466.21; found: 466.74.

4.1.3.9. Amide 10. Following the general procedure described for the coupling of amines with the Fmoc-AEEA-OH spacer, amine **9** (1.0 equiv, 30 mg, 60 μmol) provided, after purification by column chromatography (SiO_2, 95/5 CH_2Cl_2/MeOH), amide **10** (60 mg, quant. over two steps) as a yellow oil and as *E/Z* mixture of oximes. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.75 (d, J = 7.5 Hz, 4H), 7.59 (d, J = 7.5 Hz, 4H), 7.39 (dd, J = 7.5, 7.5 Hz, 4H), 7.29 (d, J = 7.5 Hz, 4H), 6.98 (d, J = 8.4 Hz, 2H), 6.53 (d, J = 8.4 Hz, 4H), 6.43 (s, 2H), 6.28 (s, 2H), 5.64 (d, J = 10.0 Hz, 2H), 5.14 (d, J = 12.2 Hz, 2H), 4.86 (d, J = 10.6 Hz, 2H), 4.40–4.35 (m, 6H), 4.21 (t, J = 6.3 Hz, 2H), 4.0 (s, 4H), 3.96 (d, J = 2.4 Hz, 2H), 3.82–3.72 (m, 4H), 3.77 (s, 6H), 3.67 (b, 4H), 3.62 (b, 4H), 3.54 (b, 4H), 3.50–3.41 (b, 4H), 3.45 (s, 6H), 3.37 (b, 4H), 1.97–1.88 (m, 4H), 1.40 (s, 12H) ppm (4× NH signal not visible); LC–MS (t_R = 2.11 min) $_{m/z}$ $[M]^+$ calcd for $C_{47}H_{51}N_3O_{11}$: 833.35; found: 833.46.

4.1.3.10. Biotin conjugate 11. Following the general procedure described for the coupling of amines with biotin *N*-hydroxysuccinimide ester, protected amine **10** (1.0 equiv, 36 mg, 43 μmol) afforded, after purification by column chromatography (C_{18}-SiO_2, from 100% H_2O to 60/40 CH_3CN/H_2O), biotinylated amide **11** (30 mg, 83%) as a white solid and as *E/Z* mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.23 (app. q, J = 6.3 Hz, 2H), 7.01 (d, J = 8.4 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 6.71 (b, 2H), 6.55 (d, J = 8.4 Hz, 2H), 6.54 (d, J = 10.0 Hz, 2H), 6.43 (s, 2H), 6.29 (b, 2H), 6.28 (s, 2H), 5.67 (d, J = 10.0, 2H), 5.39 (b, 2H), 5.19 (dd, J = 10.8, 1.6 Hz, 2H), 4.90 (dd, J = 10.8, 1.0 Hz, 2H), 4.46 (b, 2H), 4.38 (dd, J = 12.4, 2.4 Hz, 2H), 4.26 (b, 2H), 4.00 (s, 4H), 3.96 (dd, J = 12.4, 2.0 Hz, 2H), 3.77 (s, 6H), 3.75–3.39 (m, 24H), 3.46 (s, 6H), 3.10 (dd, J = 7.0, 4.7 Hz, 2H), 2.87 (dd, J = 12.8, 4.7 Hz, 2H), 2.69 (dd, J = 12.8, 3.2 Hz, 2H), 2.18 (t, J = 6.3 Hz, 4H), 2.00–1.87 (m, 4H), 1.72–1.58 (m, 8H), 1.41 (s, 6H), 1.41–1.35 (m, 4H), 1.40 (s, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 23 °C): δ 173.4 (×2), 170.1 (×2), 163.7 (×2), 158.2, 158.1 155.6 (×2), 153.3 (×2), 149.7 (×2), 148.8 (×2), 143.9 (×2), 131.3 (×2), 130.5 (×2), 116.3 (×2), 115.3 (×2), 115.0 (×2), 112.8 (×2), 111.0 (×2), 109.6 (×2), 101.5 (×2), 79.2 (×2), 76.5 (×2), 73.2 (×2), 70.9 (×2), 70.6 (×2), 70.0 (×4), 66.0 (×2), 61.7 (×2), 60.1 (×2), 55.8 (×4), 55.5 (×2), 47.8 (×2), 40.5 (×2), 39.0 (×2), 36.0 (×2), 35.7 (×2), 29.9 (×2), 28.0 (×2), 27.9 (×2), 27.7 (×2), 25.5 (×2) ppm; LC–MS (t_R = 1.55 min) m/z $[M]^+$ calcd for $C_{42}H_{55}N_5O_{11}S$: 837.36; found: 837.55. HRMS (MALDI) m/z $[M+H]^+$ calcd for $C_{42}H_{56}N_5O_{11}S$: 838.3691; found: 838.3617.

4.1.3.11. Phenols 12 and 13. BBr_3 (1.0 equiv, 200 μL, 0.20 mmol) was added to a solution of deguelin (1.0 equiv, 40 mg, 0.10 mmol) in CH_2Cl_2 (1.5 mL) at −10 °C. After stirring for 15 min at this temperature, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 75/25 petroleum ether/EtOAc), to afford phenol **13** (10 mg, 25%) and phenol **12** (20 mg, 50%) as yellow solids. *Phenol* **13**. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.74 (d, J = 8.7 Hz, 1H), 6.76 (s, 1H), 6.64 (d, J = 10.1 Hz, 1H), 6.50 (s, 1H), 6.45 (d, J = 8.7, 1H), 5.60 (b, 1H), 5.55 (d, J = 10.1 Hz, 1H), 4.90 (dd, J = 3.1, 3.1 Hz, 1H), 4.62 (dd, J = 12.1, 3.1 Hz, 1H), 4.17 (d, J = 12.1 Hz, 1H), 3.82 (d, J = 4.1 Hz, 1H), 3.77 (s, 3H), 1.45 (s, 3H), 1.38 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 23 °C): δ 189.4, 160.1, 157.0, 147.8, 146.1, 141.6, 128.6, 128.5, 115.7, 112.7, 111.4, 109.8, 109.1, 104.6, 103.5, 77.7, 72.4, 66.1, 56.4, 44.4, 28.5, 28.1 ppm. LC–MS (t_R = 1.80 min) m/z $[M]^+$ calcd for $C_{22}H_{20}O_6$:

380.13; found: 380.85. *Phenol* **12**. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.73 (d, J = 8.7 Hz, 1H), 6.83 (s, 1H), 6.63 (d, J = 10.1 Hz, 1H), 6.43 (d, J = 8.7, 1H), 6.42 (s, 1H), 5.54 (d, J = 10.1 Hz, 1H), 5.29 (b, 1H), 4.89 (dd, J = 3.0, 3.0 Hz, 1H), 4.61 (dd, J = 12.0, 3.1 Hz, 1H), 4.17 (d, J = 12.0 Hz, 1H), 3.80 (d, J = 6.1 Hz, 1H), 3.79 (s, 3H), 1.44 (s, 3H), 1.37 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 23 °C): δ 189.0, 159.9, 156.8, 146.9, 146.7, 140.1, 128.6 (×2), 115.7, 113.1, 112.7, 111.4, 109.0, 105.8, 100.1, 77.6, 72.3, 66.2, 55.8, 44.3, 28.4, 28.1 ppm. LC–MS (t_R = 1.80 min) m/z $[M]^+$ calcd for $C_{22}H_{20}O_6$: 380.13; found: 380.85.

4.1.3.12. Compound 16. Sodium borohydride (4.0 equiv, 4.0 mg, 0.10 mmol) was added to a stirring solution of phenol **13** (1.0 equiv, 10 mg, 30 μmol) in MeOH (1.0 mL) at 0 °C. After stirring for 15 min at this temperature, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.01 (d, J = 8.2 Hz, 1H), 6.69 (s, 1H), 6.66 (d, J = 9.9 Hz, 1H), 6.52 (s, 1H), 6.43 (d, J = 8.2, 1H), 5.58 (d, J = 9.9 Hz, 1H), 4.88 (d, J = 3.4 Hz, 1H), 4.83 (m, 1H), 4.57 (dd, J = 10.5, 10.5 Hz, 1H), 4.22 (dd, J = 9.6, 5.0 Hz, 1H), 3.67 (s, 3H), 3.37 (dd, J = 5.3, 4.5 Hz, 1H), 1.44 (s, 3H), 1.42 (s, 3H) ppm (2 OH signals are not visible). LC–MS (t_R = 1.71 min) m/z $[M-OH]^+$ calcd for $C_{22}H_{21}O_5$: 365.14; found: 365.20.

A solution containing the above phenol (1.0 equiv, 14 mg, 40 μmol), 1-azido-3-iodopropane (1.5 equiv, 12 mg, 50 μmol) and K_2CO_3 (3.0 equiv, 51 mg, 0.11 mmol) in DMF (0.5 mL) was stirred for 48 h at 23 °C. The reaction was quenched with water (1.0 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.02 (d, J = 8.3 Hz, 1H), 6.73 (s, 1H), 6.66 (d, J = 10.0 Hz, 1H), 6.48 (s, 1H), 6.43 (d, J = 8.3 Hz, 1H), 5.58 (d, J = 10.0 Hz, 1H), 4.90 (d, J = 3.7 Hz, 1H), 4.88–4.82 (m, 1H), 4.59 (dd, J = 11.0, 9.9 Hz, 1H), 4.24 (ddd, J = 9.9, 5.2, 1.0 Hz, 1H), 4.07 (t, J = 6.3 Hz, 2H), 3.84 (s, 3H), 3.54 (t, J = 6.3 Hz, 2H), 3.38 (dd, J = 5.2, 3.7 Hz, 1H), 2.09 (quint, J = 6.3 Hz, 2H), 1.44 (s, 3H), 1.42 (s, 3H) (OH signal is not visible) ppm. LC–MS (t_R = 2.03 min) m/z $[M-OH]^+$ calcd for $C_{25}H_{26}N_3O_5$: 448.19; found: 448.18.

Pyridinium dichromate (1.5 equiv, 24 mg, 60 μmol) was added to a stirring solution of the above alcohol (1.0 equiv, 20 mg, 40 μmol) and 4 Å molecular sieves (0.5 g/mmol) in CH_2Cl_2 (1.0 mL) at 23 °C. After stirring for 15 min at this temperature, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, from 70/30 petroleum ether/EtOAc), to afford ketone **16** (12 mg, 54% over three steps) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.75 (d, J = 8.7 Hz, 1H), 6.80 (s, 1H), 6.65 (d, J = 10.1 Hz, 1H), 6.46 (s, 1H), 6.45 (d, J = 8.7, 1H), 5.56 (d, J = 10.1 Hz, 1H), 4.91 (t, J = 3.0 Hz, 1H), 4.61 (dd, J = 12.0, 3.1 Hz, 1H), 4.18 (d, J = 12.0 Hz, 1H), 4.06–3.99 (m, 2H), 3.83 (d, J = 4.0 Hz, 1H), 3.75 (s, 3H), 3.50 (dt, J = 6.7, 1.5 Hz, 2H), 2.05 (quint, J = 6.6 Hz, 2H), 1.45 (s, 3H), 1.39 (s, 3H) ppm; LC–MS (t_R = 2.11 min) m/z $[M+H]^+$ calcd for $C_{25}H_{25}N_3O_6$: 464.18; found: 464.10.

4.1.3.13. Biotin conjugate 17. Following the general procedure described for the reduction of azides, compound **16** (1.0 equiv, 16 mg, 30 μmol) gave amine as a yellow oil, which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.74 (d, J = 8.7 Hz, 1H), 6.79 (s, 1H), 6.64 (d, J = 10.0 Hz, 1H), 6.46 (s, 1H), 6.45 (d, J = 8.7 Hz, 1H), 5.56 (d, J = 10.0 Hz, 1H), 4.91 (dd, J = 3.1, 3.1 Hz, 1H), 4.62 (dd, J = 12.0, 3.1 Hz, 1H), 4.18 (d, J = 12.0 Hz, 1H), 4.07–4.00 (m, 2H), 3.85–3.82 (m, 1H), 3.74 (s, 3H), 2.89 (t, J = 6.3 Hz, 2H), 1.94 (t, J = 6.3 Hz, 2H), 1.45 (s, 3H), 1.39 (s, 3H) ppm (NH_2 signal is not visible); LC–MS (t_R = 1.37 min) m/z $[M+H]^+$ calcd for $C_{25}H_{28}NO_6$: 438.19; found: 438.31.

Following the general procedure described for the coupling of amines with Fmoc-AEEA-OH spacer, above amine (1.0 equiv, 16 mg, 40 μmol) provided, after purification by column chromatography (SiO_2, 95/5 CH_2Cl_2/MeOH), the desired amide (20 mg, 71% over two steps) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.80–7.72 (m, 3H), 7.60–7.55 (m, 2H), 7.40–7.35 (m, 2H), 7.33–7.26 (m, 2H), 6.78 (s, 1H), 6.63 (d, J = 10.5 Hz, 1H), 6.44 (d, J = 8.9 Hz, 1H), 6.43 (s, 1H), 5.55 (d, J = 10.1 Hz, 1H), 4.90 (b, 1H), 4.59 (d, J = 12.0 Hz, 1H), 4.42–4.40 (m, 2H), 4.16–4.13 (m, 3H), 3.97 (s, 2H), 3.82 (b, 1H), 3.74 (b, 1H), 3.69 (s, 3H), 3.64 (b, 2H), 3.57 (b, 2H), 3.47 (b, 4H), 3.33 (b, 2H), 2.00–1.92 (m, 2H), 1.45 (s, 3H), 1.37 (s, 3H) ppm (2 NH signals are not visible); LC–MS (t_R = 2.14 min) m/z $[M+H]^+$ calcd for $C_{46}H_{49}N_2O_{11}$: 805.33; found: 805.25.

Following the general procedure described for the coupling of amines with biotin *N*-hydroxysuccinimide ester, above compound (1.0 equiv, 20 mg, 30 μmol) afforded, after purification by HPLC (Zorbax C18, 9.4 cm × 25 mm, 5 μm particle size, gradient from 40% CH_3CN in 60% H_2O with 0.01% TFA to 80% CH_3CN in 20% H_2O with 0.01% TFA, 3 mL/min, over 20 min, t_R = 7.65 min), biotinylated amide **17** (6 mg, 30%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.74 (d, J = 8.7 Hz, 1H), 6.81 (s, 1H), 6.64 (d, J = 10.1 Hz, 1H), 6.46 (s, 1H), 6.45 (d, J = 8.7 Hz, 1H), 5.57 (d, J = 10.1 Hz, 1H), 4.92 (b, 1H), 4.63 (dd, J = 12.1, 2.2 Hz, 1H), 4.53 (b, 1H), 4.34 (b, 1H), 4.19 (d, J = 12.0 Hz, 1H), 4.06–4.00 (m, 2H), 4.00 (s, 2H), 3.84 (d, J = 3.9 Hz, 1H), 3.74 (s, 3H), 3.65 (b, 2H), 3.57 (b, 2H), 3.49 (b, 4H), 3.44–3.38 (m, 2H), 3.15 (b, 1H), 2.95–2.85 (m, 1H), 2.72 (dd, J = 12.6, 6.2 Hz, 1H), 2.05–1.98 (m, 2H), 1.75–1.56 (m, 6H), 1.45 (s, 3H), 1.45–1.41 (m, 2H), 1.39 (s, 3H) ppm (4 NH signals are not visible); LC–MS (t_R = 1.58 min) m/z $[M+H]^+$ calcd for $C_{41}H_{53}N_4O_{11}S$: 809.34 found: 809.44. HRMS (MALDI) m/z $[M+H]^+$ calcd for $C_{41}H_{53}N_4O_{11}S$: 809.3426; found: 809.3413.

4.1.3.14. Ketone 14. Sodium borohydride (4.0 equiv, 14 mg, 0.38 mmol) was added to a stirring solution of phenol **12** (1.0 equiv, 36 mg, 90 μmol) in MeOH (1.0 mL) at 0 °C. After stirring for 15 min at this temperature, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.01 (d, J = 8.3 Hz, 1H), 6.78 (s, 1H), 6.66 (d, J = 9.9 Hz, 1H), 6.46 (s, 1H), 6.43 (d, J = 8.3 Hz, 1H), 5.58 (d, J = 9.9 Hz, 1H), 4.86 (d, J = 3.6 Hz, 1H), 4.86–4.82 (m, 1H), 4.58 (dd, J = 10.8, 9.9 Hz, 1H), 4.22 (dd, J = 9.6, 5.2 Hz, 1H), 3.86 (s, 3H), 3.37 (dd, J = 5.3, 4.9 Hz, 1H), 1.43 (s, 3H), 1.42 (s, 3H) ppm (2 OH signals are not visible). LC–MS (t_R = 1.70 min) m/z $[M-OH]^+$ calcd for $C_{22}H_{21}O_5$: 365.14; found: 365.33.

A solution containing the crude above phenol (1.0 equiv, 27 mg, 70 μmol), 1-azido-3-iodopropane (1.5 equiv, 22 mg, 0.11 mmol) and potassium carbonate (3.0 equiv, 30 mg, 0.21 mmol) in DMF (0.5 mL) was stirred for 48 h at 23 °C. The reaction was quenched with water (1.0 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.01 (d, J = 8.3 Hz, 1H), 6.78 (s, 1H), 6.65 (d, J = 10.0 Hz, 1H), 6.46 (s, 1H), 6.41 (d, J = 8.3 Hz, 1H), 5.57 (d, J = 10.0 Hz, 1H), 4.88 (d, J = 3.8 Hz, 1H), 4.88–4.80 (m, 1H), 4.59 (dd, J = 10.0, 10.0 Hz, 1H), 4.22 (dd, J = 8.9, 4.3 Hz, 1H), 4.04

(t, J = 6.3 Hz, 2H), 3.81 (s, 3H), 3.54 (t, J = 6.3 Hz, 2H), 3.36 (d, J = 5.1 Hz, 1H), 2.03 (quint, J = 6.3 Hz, 2H), 1.42 (s, 3H), 1.40 (s, 3H) (OH signal not visible) ppm. LC–MS (t_R = 2.06 min) m/z $[M-OH]^+$ calcd for $C_{25}H_{26}N_3O_5$: 448.19; found: 448.17.

Pyridinium chromate (1.5 equiv, 85 mg, 0.22 mmol) was added to a stirring solution of above alcohol (1.0 equiv, 70 mg, 0.15 mmol) and 4 Å molecular sieves (0.5 g/mmol) in CH_2Cl_2 (1.0 mL) at 23 °C. After stirring for 15 min at this temperature, the reaction was quenched with water (1.5 mL) and extracted with EtOAc (3 × 2.0 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, from 70/30 petroleum ether/EtOAc), to afford ketone **14** (40 mg, 70% over three steps) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.74 (d, J = 8.7 Hz, 1H), 6.82 (s, 1H), 6.64 (d, J = 10.1 Hz, 1H), 6.46 (d, J = 8.7 Hz, 1H), 6.45 (s, 1H), 5.56 (d, J = 10.1 Hz, 1H), 4.91 (b, 1H), 4.63 (dd, J = 12.0, 2.9 Hz, 1H), 4.19 (d, J = 12.0 Hz, 1H), 3.97 (m, 2H), 3.82 (d, J = 4.2 Hz, 1H), 3.79 (s, 3H), 3.49 (t, J = 6.3 Hz, 2H), 2.00 (quint, J = 6.3 Hz, 2H), 1.45 (s, 3H), 1.39 (s, 3H) ppm. LC–MS (t_R = 2.12 min) m/z $[M+H]^+$ calcd for $C_{25}H_{26}N_3O_6$: 464.17; found: 463.96.

4.1.3.15. Biotin conjugate 15. Following the general procedure described for the reduction of azides, azide **14** (1.0 equiv, 40 mg, 90 μmol) gave the desired amine as a yellow oil, which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.74 (d, J = 8.7 Hz, 1H), 6.82 (s, 1H), 6.64 (d, J = 10.2 Hz, 1H), 6.45 (d, J = 8.7 Hz, 1H), 6.44 (s, 1H), 5.56 (d, J = 10.2 Hz, 1H), 4.91 (b, 1H), 4.63 (dd, J = 12.1, 3.5 Hz, 1H), 4.19 (d, J = 12.1 Hz, 1H), 4.01–3.94 (m, 2H), 3.90–3.80 (m, 1H), 3.78 (s, 3H), 2.87 (t, J = 6.3 Hz, 2H), 1.89 (t, J = 6.3 Hz, 2H), 1.45 (s, 3H), 1.39 (s, 3H) ppm (NH_2 signal is not visible); LC–MS (t_R = 1.34 min) m/z $[M+H]^+$ calcd for $C_{25}H_{28}NO_6$ 438.19; found: 438.27.

Following the general procedure described for the coupling of amines with Fmoc-AEEA-OH spacer, the above amine (1.0 equiv, 35 mg, 0.08 mmol) provided, after purification by column chromatography (SiO_2, 95/5 CH_2Cl_2/MeOH), the desired protected amide (50 mg, 70% over two steps) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.80–7.72 (m, 3H), 7.60–7.65 (m, 2H), 7.40–7.35 (m, 2H), 7.33–7.26 (m, 2H), 7.18 (b, 1H), 6.81 (b, 1H), 6.63 (d, J = 10.1 Hz, 1H), 6.44 (d, J = 8.9 Hz, 1H), 6.43 (s, 1H), 5.51 (d, J = 10.1 Hz, 1H), 5.47 (b, 1H), 4.89 (b, 1H), 4.61 (dd, J = 12.0, 3.0 Hz, 1H), 4.39–4.37 (m, 2H), 4.24–4.13 (m, 3H), 3.98 (s, 2H), 3.97–3.91 (m, 1H), 3.80 (dd, J = 15.0, 3.9 Hz, 1H), 3.73 (s, 3H), 3.64 (b, 2H), 3.56 (b, 2H), 3.50–3.42 (m, 4H), 3.33 (b, 2H), 1.94 (t, J = 6.3 Hz, 2H), 1.44 (s, 3H), 1.37 (s, 3H) ppm; LC–MS (t_R = 2.14 min) m/z $[M+H]^+$ calcd for $C_{46}H_{49}N_2O_{11}$ 805.33; found: 805.27.

Following the general procedure described for the coupling of amines with biotin *N*-hydroxysuccinimide ester, the protected above amine (1.0 equiv, 48 mg, 30 μmol) afforded, after purification by HPLC (Zorbax C18, 9.4 cm × 25 mm, 5 μm particle size, gradient from 40% CH_3CN in 60% H_2O with 0.01% TFA to 80% CH_3CN in 20% H_2O with 0.01% TFA, 3 mL/min, over 20 min, t_R = 7.58 min), biotinylated amide **15** (14 mg, 31%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C): δ 7.72 (d, J = 8.7 Hz, 1H), 6.83 (s, 1H), 6.64 (d, J = 10.1 Hz, 1H), 6.46 (s, 1H), 6.45 (d, J = 8.7 Hz, 1H), 5.57 (d, J = 10.1 Hz, 1H), 4.92 (b, 1H), 4.64 (d, J = 9.4 Hz, 1H), 4.54 (b, 1H), 4.35 (b, 1H), 4.20 (d, J = 9.4 Hz, 1H), 4.02 (s, 2H), 4.01–3.95 (m, 2H), 3.83 (d, J = 3.0 Hz, 1H), 3.78 (s, 3H), 3.65 (b, 2H), 3.57 (b, 2H), 3.48 (b, 4H), 3.39 (b, 2H), 3.15 (b, 1H), 2.91 (dd, J = 12.6, 4.2 Hz, 1H), 2.73 (d, J = 12.6 Hz, 1H), 1.96 (t, J = 6.3 Hz, 2H), 1.68–1.62 (m, 6H), 1.45 (s, 3H), 1.45–1.40 (m, 2H), 1.39 (s, 3H) (4 NH signals are not visible) ppm; HRMS (MALDI) m/z $[M+H]^+$ calcd for $C_{41}H_{53}N_4O_{11}S$: 809.3426; found: 809.3411.

4.2. Biology

In vivo tubulin polymerization inhibition and fluorescence staining. PtK_2 cells (ATCC CCL-56) were grown on glass coverslips (13 mm diameter) in four-well plates. Exponentially growing cells were incubated with the compounds for 16 h. Cells were fixed with cold (−20 °C) acetone/methanol (1:1) for 10 min. For labeling the microtubules, cells were incubated with a primary monoclonal antibody against α-tubulin (1:500; Sigma), then with a secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:200; Invitrogen) at 37 °C for 45 min. The nuclei and chromosomes were stained with DAPI (1 μg/ml). The cells were washed with PBS between different incubations. The coverslips were mounted using Prolong Antifade (Molecular Probes), and viewed with a Zeiss Axiophot fluorescence microscope using appropriate filter sets.

Tubulin purification and polymerization assay. Microtubule proteins were purified from porcine brain homogenates using standard procedures that comprise two to three cycles of temperature-dependent polymerization and depolymerization.[24] Microtubule proteins are composed of tubulin and MAPs (microtubule-associated proteins). Tubulin polymerization was monitored via turbidometry.[25] 200 μL of microtubule proteins (1.25 mg/mL) in PEM polymerization buffer (0.1 M PIPES, pH 6.6, 1 mM $MgSO_4$, 1 mM EGTA and 1 mM GTP) was rapidly warmed to 37 °C in a water jacketed cuvette holder of a diode array photometer (Beckman Spectrophotometer DU 7500). The absorbance at 350 nm was monitored in presence of different drug concentrations and the vehicle only.

4.2.1. Cell culture and mitochondria staining

MCF7 cells were cultured in DMEM supplemented with 10% FBS, 1% L-Glutamin and 1% PenStrep at 37 °C in humidified atmosphere containing 5% CO_2. Cells were seeded on 35 mm glass bottom dishes 12 h before the experiment. Then, they were incubated for 45 min with 700 nM MitoTracker Red CM-H_2XRos (Molecular Probes) in PBS under growth conditions to achieve mitochondria staining. After cells were washed with pre-warmed PBS and incubated with fresh growth medium for an additional hour before fixation.

4.2.2. Cellular localization of deguelin conjugates

Cells, washed once with PBS, were fixed with 3.7% freshly prepared formaldehyde in PBS (15 min, rt). After washing with PBS (3 × 5 min, rt) they were permeabilized with 0.1% Triton X-100 in PBS for 4 min and washed with PBS (3 × 2 min, rt). Compounds **5**, **11**, **15**, **17** were dissolved in DMSO to obtain a 10 mM solutions and 2.5 μL of these solutions were pre-incubated with 50 μL of streptavidin-FITC (1 mg/mL) at 4 °C for 1 h. Then non protein-bound fraction was removed using 30 kDa spin filters (Vivaspin 500). Remaining material was diluted with PBS (estimated final concentration 50 μM) and added for 2 h to fixed and permeabilized cells with stained mitochondria. Then, the incubation solution was removed, cells were washed with PBS and treated with 250 nM DAPI for 5 min to stain the nucleus. After final washing with PBS cells were imaged with 60× oil immersion objective using a Nikon Eclipse Ti inverted fluorescence microscope with xenon excitation source (for DAPI filter set: excitation 325–375 nm, emission 435–485 nm; FITC: excitation 460–500 nm, emission 510–560 nm; MitoTracker Red: excitation 533–588 nm, emission 608–683 nm). Images were acquired and analyzed using NIS Elements Advance Research Software. Colocalization between signals from different channels was evaluated using integrated measurement module. Intensity profiles and populations' distribution for each color pair were compared and Pearson's correlation as well as Mander's overlap values were calculated.

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