Indolizine-Based Scaffolds as Efficient and Versatile Tools: Application to the Synthesis of Biotin-Tagged Antiangiogenic Drugs

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** Supporting Information

ABSTRACT: We describe the design and optimization of polyfunctional scaffolds based on a fluorescent indolizine core derivatized with various orthogonal groups (amines, esters, oximes, alkynes, etc.). To show one application as tools in biology, the scaffold was used to prepare drug–biotin conjugates that were then immobilized onto avidin-agarose for affinity chromatography. More specifically, the antiangiogenic drug COB223, whose mechanism of action remained unclear, was chosen as a proof-of-concept drug. The drug-selective discrimination of proteins observed after elution of the cell lysates through the affinity columns, functionalized either with the biologically active COB223 or a structurally related inactive analogue (COB236), is a clear indication that the presence of the indolizine core does not limit drug–protein interaction and confirms the usefulness of the indolizine scaffold. Furthermore, the separation of COB223-interacting proteins from human placental extracts unveiled unanticipated protein targets belonging to the family of regulatory RNA-binding proteins, which opens the way to new hypotheses on the mode of action of this antiangiogenic drug.

INTRODUCTION

During the last decades, the development of orthogonal chemistries1,2 opened the way to the conception of molecular scaffolds decorated with orthogonal reactive groups (azide, alkyne, alkene, carbonyl, etc.) and their use for bioconjugations.3−7 So far, there are a few heterocyclic platforms,8−10 and none with intrinsic luminescent properties. In a previous work, we considered the use of pyridinium ylide-alkyne cycloaddition reactions to prepare drug–biotin conjugates that were then immobilized onto avidin-agarose for affinity chromatography. More specifically, the antiangiogenic drug COB223, whose mechanism of action remained unclear, was chosen as a proof-of-concept drug. The drug-selective discrimination of proteins observed after elution of the cell lysates through the affinity columns, functionalized either with the biologically active COB223 or a structurally related inactive analogue (COB236), is a clear indication that the presence of the indolizine core does not limit drug–protein interaction and confirms the usefulness of the indolizine scaffold. Furthermore, the separation of COB223-interacting proteins from human placental extracts unveiled unanticipated protein targets belonging to the family of regulatory RNA-binding proteins, which opens the way to new hypotheses on the mode of action of this antiangiogenic drug.

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complicated isolation and purification. Designing an original synthetic procedure for biotin-tagged drugs based on the indolizine scaffold was an appealing option. We report here the chemistry of 7-acetyl- and 7-carboxamidoindolizines as potential multifunctional platforms. Various orthogonal groups (esters, amines, alkoxyamines, alkynes) were introduced at positions 1, 3, and 7 of the indolizine rings, by either pre- or postfunctionalization to illustrate the variety of reactions that may be performed. The most suitable methodology was applied to the synthesis of two biotin-tagged antiangiogenic drugs. To evaluate the possible impact of the indolizine nucleus on drug−protein interaction, the biotin-tagged molecules were immobilized onto streptavidin agarose beads and used for affinity chromatography and subsequent proteomics analysis. The comparison of the data obtained using either the biologically active drug (COB223) or the inactive analogue (COB236) is discussed.

RESULTS AND DISCUSSION
The indolizines shown in Scheme 1 contain three points of modification, each of them coming from one of the three reactants: R1 from the pyridine, R2 from the alkylating agent (halogeno acetic ester or amide), and R3 from the activated alkyne (propiolic ester or amide). Two strategies were envisioned for the introduction of functional groups: either before cyclization (prefunctionalization) by synthesizing modified starting reactants, or after cyclization (more versatile postfunctionalization). Choosing between the two approaches would mainly be dependent on the stability and orthogonality of the different functions, and on the efficiency of isolation and purification.

7-Acetyl Indolizine 3 was first evaluated as a potential tripodal scaffold (Scheme 2A) with methyl ester and two “clickable” functions (alkyne and carbonyl) for further postfunctionalization. The triple bond may either be introduced using the propiolic amide 2 as dipolarophile or via formation of the reactive para-nitrophenyl ester 5 followed by substitution with propargylamine. Next, the reaction with alkoxyamines (illustrated by benzylxoyamine) yielded the corresponding oxime 4.

In this first example, the amino group (i.e., propargylamine) was introduced at position 1 of the indolizine ring; however, it was also valuable to link amino groups to other positions. Indolizines 8, 9, and 12 contain the amino groups (illustrated by propylene, see Scheme 2B) at position 3. To do so, the corresponding 2-bromo-N-propylacetamide was used as alkylating agent in the preparation of the pyridinium salt (7 or 11). Two strategies were then evaluated. To form the indolizine 8, the rather stable ethyl ester was introduced at position 1 by reacting ethyl propiolate with 7. The oxime bond was later formed to give 9. In the case of indolizine 12 bearing reactive para-nitrophenyl ester, the oxime bond was formed in excellent yield at an earlier stage, i.e., before alkylation with the 2-bromo-N-propylacetamide and cyclization with the para-nitrophenyl propionate 5. Lastly, the amino group (exemplified by the mono-Boc-protected 2,2′-(ethylenedioxy)bis(ethylamine), often used as linker in the synthesis of bioconjugates), was introduced at position 7 of the indolizine ring. As drawn in Scheme 2C, isonicotinic amide 14 was prepared from the activated ester 13 and mono-Boc-protected 2,2′-(ethylenedioxy)bis(ethylamine). Alkylation with the ethyl 2-bromoacetate gave the pyridinium salt 15a. To highlight the high reactivity of ester at position 1 of pyridinium salts, due to the presence of the positive charge at β-position, a trans-esterification was realized in CD3OD at room temperature (rt) with the formation of the deuterated analogue 15b. We thus formed a reactive scaffold containing two esters of different
stabilities, and a Boc-protected amine that can easily be released by trifluoroacetic acid (TFA) treatment.

The latter approach was chosen to prepare the two biotin-tagged COB223 and COB236. Indeed, direct binding of biotin to the molecule was precluded due to limited access of the drug by the target protein during affinity chromatography, and to overcome this problem, ethylene glycol (EG) linker such as in Biot-NH-EG-NH₂ (Scheme 3) was added. The site of functionalization of the active drug by the biotin was another key point to examine. As reported earlier, the structure–activity relationships pointed to the importance of both the dansyl chromophore and the polyamine linker for the antiangiogenic properties. We therefore chose to modify the Boc group of COB223 and COB236. As depicted in Scheme 3, the drugs were introduced at the last step to limit tedious purification steps. The key intermediate, Biot-NH-EG-NH-Py, was prepared from Biot-NH-EG-NH₂ by reaction with the reactive para-nitrophenyl isonicotinic ester. Alkylation with methyl 2-bromoacetate in acetone gave the corresponding pyridinium bromide in excellent yield. This intermediate was stable at room temperature for extended period of time. Cyclization with para-nitrophenyl propiolate in dimethylformamide (DMF) in the presence of K₂CO₃ gave the indolizine in reasonable yield. Due to the reactivity of the para-nitrophenyl ester, the next step was performed without further purification. The nucleophilic substitution by the primary amines of 21a,b, issued from the Boc deprotection of COB233.

Scheme 2. Different Strategies of Formation and Modulation of Tripodal Core. Amine Containing Reactants were Introduced at Positions 1 (Part A), 3 (Part B) or 7 (Part C) of the Indolizine Ring.

A

B

C

stabilities, and a Boc-protected amine that can easily be released by trifluoroacetic acid (TFA) treatment.

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or COB236, yielded the corresponding biotin-tagged molecules.

COB223-biotin and COB236-biotin were then immobilized onto streptavidin agarose beads. We had observed during the synthesis that COB223-biotin and COB236-biotin were very stable in organic solvents, and to test the stability in aqueous solutions, the samples of the modified gels were suspended in a pH 7.5 buffer that was then used for affinity chromatography. The suspensions were shaken several days at various temperatures (40°–70° C). Thanks to the fluorescence properties of the indolizine core, the gels and supernatants obtained after centrifugation were easily analyzed under UV−vis irradiation. The gels remained highly fluorescent, and no detectable trace of fluorescent molecules was observed in the supernatants (see Figure S1), confirming the stability of COB−biotin−streptavidin columns in conditions much harsher than those routinely used in biology.

As COB223 was previously shown to be antiangiogenic, whereas the structurally related COB236 was inactive in angiogenesis assays, we selected a highly vascularized tissue, the placenta, as a source of target proteins. Affinity purifications of COB-binding proteins were then performed by loading 400 mg of placental extract proteins (in Tris 20 mM-HCl pH 7.5, NaCl 75 mM, Triton X-100 0.05%) onto each column. Experiments were performed at 4 °C in the presence of protease inhibitors (Sigma) to prevent any degradation not only of the proteins but also of the drug−biotin conjugates. The columns were washed with 5 volumes of Tris 20 mM-HCl pH 7.5 and then eluted successively with 3 column volumes of Tris 20 mM-HCl pH 7.5 0.2 M NaCl and then with 3 column volumes of Tris 20 mM-HCl pH 7.5, 0.5 M NaCl. The 0.5 M NaCl eluates of each column were concentrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in stacking polyacrylamide gels, which were then trypsinized and the released peptides were analyzed by liquid chromatography−tandem mass spectrometry (LC−MS/MS). This allowed us to identify 40 proteins that were significantly more abundant in COB223-agarose eluates than in COB236-agarose eluates (Table S1). Although we previously reported that COB223 inhibits VEGF receptor 2 (VEGFR2) activation of ERK1/2 phosphorylation in microvascular endothelial cells,¹⁹ none of these COB223-binding proteins was a known intermediate of the VEGF signaling pathway. Then, using the DAVID Bioinformatics Resource (v 6.8; NIAID, NIH), we searched to which pathway these proteins belonged in majority. It appeared that 13 out of 38 members (2 out of 40 were not referenced in DAVID) of this protein cluster (listed in Table 1)
were annotated as poly(A) RNA-binding proteins, corresponding to a 4.7-fold enrichment (p value = 1.9 × 10^{-5} corresponding to 2.0 × 10^{-3} after Benjamini–Hochberg correction for multiple testing). The procedure, called gene ontology enrichment, searches for biological functions, processes, and localizations that are over-represented in the 38 “hit proteins” as compared to a random selection of 38 proteins among translated sequences from the whole human genome.

Such a low p value indicates a significant enrichment of poly(A) RNA-binding proteins among the proteins binding specifically to COB223. These included two ribosomal proteins (S14, S20) and several proteins known to regulate RNA translation, splicing, or stability through tight protein–RNA interactions. We thus speculate that COB223 might interfere with the RNA-processing machinery and thereby modify the level of expression of essential components of the VEGF signaling pathway. The most differentially retained RNA-binding protein, PA2G4, is of great interest, as it is also known as ErbB3-binding protein (EBP1). EBP1 has been implicated in growth inhibition and the induction of differentiation of human cancer cells. Whether it can also interact with VEGF receptors and the VEGF signaling pathway will be worth testing in the future.

**CONCLUSIONS**

As a conclusion, we have developed the chemistry of 1,3,7-trifunctionalized indolizines toward their uses as di- or tripod scaffolds. This approach has several advantages, and in particular commercially or easily accessible reactants and mild reaction conditions. Various functional groups (esters, amines, alkynes, oxazines, carbonyls, etc.) were successfully introduced on the scaffold by combining pre- or postfunctionalization reactions, ensuring the versatility of this platform. Another interesting aspect is linked to the intrinsic fluorescence of the indolizine ring, allowing the easy monitoring of the molecules not only during synthesis, but also in the course of biochemical and biological assays. In the present work, fluorescence properties were applied to assess the stability of biotin-tagged molecules.

To illustrate a first application as a tool in molecular biology, we created a new biotin-modified scaffold convenient to prepare biotin-tagged drugs for cellular targets identification using proteomics analysis. The biotin-derived indolizine was synthesized in two steps, and was used as the key reagent to label amine-containing molecules. As proof of concept, the antiangiogenic COB223 drug and the inactive COB236 analogue were studied. The drug-selective discrimination of proteins observed after elution of the cell lysates through the affinity columns, functionalized either with the active (COB223) drug or its structurally related inactive (COB236) analogue, is a clear indication that the presence of the indolizine core does not limit the drug–protein interaction, thus confirming the usefulness of the new biotin–indolizine reagent for drug tagging.

Very interestingly, affinity purification of COB223-interacting proteins from human placental extracts allowed us to identify unanticipated protein targets. None of the primary signaling enzymes that contribute to the VEGFR2 signaling cascade (VEGF receptor 2, Ras, intracellular protein-kinases of the ERK, p38 MAP-kinase, and Akt families) was present among the affinity-purified proteins. In contrast, COB223 appeared to interact with several regulatory RNA-binding proteins, suggesting that it targets indirect mechanisms such as mRNA editing or mRNA stability, which in turn may affect the level of expression of some crucial components of the VEGFR2 signaling pathway. Another possibility is that COB223 acts via binding to PA2G4/EBP1 through direct interference with the VEGF receptors. These unexpected observations open the way for a whole set of new biological experiments aimed at better understanding the mechanism of action of the antiangiogenic compound COB223, but the exploration of these new hypotheses goes beyond the scope of the present publication mainly dedicated to the indolizine chemistry.

From the chemical point of view, we are now considering other applications in the fields of surface functionalization and macromolecules modifications.

**MATERIALS AND METHODS**

NMR spectra were recorded at room temperature in 5 mm tubes on a Bruker AC 400 MHz spectrometer (NMR facility, PCN-ICMG, Grenoble). Chemical shifts (δ) are reported in parts per million (ppm) from low to high field and referenced to residual nondeuterated solvent relative to Me4Si. Standard abbreviations for multiplicity were used as follows: s = singlet; d = doublet; t = triplet; m = multiplet. High-resolution mass spectrometry (HRMS) was carried out on a Bruker UHR-Q-TOF MaXis-ETD (time of flight) mass spectrometer using electrospray ionization (ESI) in Institut de Chimie Organique et Analytique (CBM-ICOA) in Orleans (France). Reversed-phase HPLC was performed with a μ-bondapak-C18 analytical column (Waters Corporation, Milford, MA). A Waters chromatographic system was used, with two M-510 pumps and a photodiode array detector Waters 996 using Millenium software. A linear gradient from 0 to 100% methanol in H2O pH 2.5 (phosphoric acid), 2 mL/min flow rate, was used.

N-(Prop-2-yn-1-yl)-prop-2-ynamide was prepared by biocatalyzed reaction as reported by us. para-Nitrophenyl propiolate, *para*-nitrophenyl isonicotinate, NH2-EG-Biotine-TFA, and dansyl and tosyl sulfonamides were prepared following reported procedures. Streptavidinagarose was purchased from Thermo Scientific Pierce (Waltham, MA).

**Syntheses.** 7-Acetyl-3-methyl-1-[(prop-2-yn-1-yl)-carbamoyljindolizine-3-carboxylate. Method 1. The 4-acetylpyridinium bromide (113 mg, 0.41 mmol) and propiolic amide (2 mg, 0.49 mmol) were dissolved in DMF (2 mL). K2CO3 (57 mg, 0.41 mmol) was then added and the mixture was stirred overnight at rt. The solid was filtered off and the solution concentrated under reduced pressure. The residue was then purified by flash chromatography on silica gel (cyclohexane/ethyl acetate: 6/4). Indolizine 3 was thus isolated as a yellow solid (30 mg, 25%).

Method 2. A suspension of propargylamine (64 μL, 1 mmol) and indolizine 6 (60 mg, 0.16 mmol) in CH2Cl2 (3 mL) was vigorously stirred during 3 days at rt. The solid was filtered off and the solution concentrated under reduced pressure. The residue was then purified by flash chromatography on silica gel (cyclohexane/ethyl acetate: 6/4). Indolizine 3 was thus isolated as a yellow solid (24 mg, 52%).
7-[[1-(Benzyloxy)imino]ethyl]-3-methyl-1-[[prop-2-yn-1-y]carbamoyl]indolizine-3-carboxylate 4. A suspension of indolizine 3 (30 mg, 0.10 mmol) and O-benzoylhydroxylamine hydrochloride (50 mg, 0.31 mmol) was stirred in MeOH (1 mL) for 48 h at 50 °C overnight and the white precipitate was filtered off and dried. The oxime ether 4 was thus obtained in 35% yield (14 mg, 0.03 mmol). 1H NMR (400 MHz, CDCl3) δ 9.39 (dd, 1H, J = 7.4, 0.9 Hz), 8.71 (m, 1H), 7.71 (s, 1H), 7.60 (dd, 1H, J = 7.4, 1.9 Hz), 7.3–7.5 (m, 5H), 6.13 (br m, 1H), 5.33 (s, 2H), 4.32 (dd, 2H, J = 5.3, 2.5 Hz), 3.98 (s, 3H), 2.38 (s, 3H), 2.36 (t, 1H, J = 2.4 Hz); 13C NMR (100 MHz, CDCl3) δ 163.7, 161.2, 152.7, 138.4, 137.7, 133.4, 128.2, 127.9, 126.6, 119.7, 117.3, 114.5, 111.8, 79.8, 77.9, 71.7, 51.5, 29.2, 11.6; HRMS (ESI) m/z: calc for C23H21N3O4 [M + Na]+ 426.1423, obsd 426.1423; calc for C23H22N3O4 [M + H]++ 404.1604, obsd 404.1604.

5-1-[(4-nitrophenyl)-3-methyl-indolizine-1,3-dicarboxylate 6. The 4-acrylpimidine bromide 1 (128 mg, 0.46 mmol) and para-nitrophenyl propionic ester 5 (134 mg, 0.7 mmol) were dissolved in DMF (1.5 mL). NaHCO3 (39 mg, 0.46 mmol) was then added and the mixture was stirred overnight at rt. The solution was then diluted with CH2Cl2 (5 mL) and the white solid (NaBr) was filtered off. Diethyl ether (10 mL) was then added to the cloudy filtrate allowing the precipitation of a light suspension that was filtered off. The resulting clear solution was concentrated under reduced pressure and poured into a large volume of diethyl ether (50 mL). The solution was allowed to stand at rt for 48 h. The resulting precipitate was filtered, washed twice with diethyl ether and dried. Indolizine 6 was thus obtained as a beige solid (74 mg, 42%). 1H NMR (400 MHz, CDCl3) δ 9.64 (dd, 1H, J = 7.2, 0.8 Hz), 8.97 (dd, 1H, J = 1.6, 0.8 Hz), 8.40 (s, 2H, J = 9.2 Hz), 8.24 (s, 1H), 7.67 (dd, 2H, J = 7.2, 2.0 Hz), 7.52 (dd, 1H, J = 9.2 Hz), 4.04 (s, 3H), 2.75 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 195.7, 161.0 (2C), 155.5, 145.4, 138.5, 133.8, 128.1, 125.3, 125.2, 122.7, 120.8, 117.1, 112.6, 106.3, 52.0, 26.2; HRMS (ESI) m/z: calc for C16H15N2O4 [M + H]+ 287.1199, obsd 287.1199; calc for C16H15N2O4 [M + H]+ 287.1199. HRMS (ESI) m/z: calc for C16H15N2O4 [M + H]+ 287.1199, obsd 287.1199; calc for C16H15N2O4 [M + Na]+ 309.1315, obsd 309.1314; calc for C20H21N2O4 [M + H]+ 317.1496, obsd 317.1494 and calc for C20H22N2O4 [M + H]+ 358.1501, obsd 358.1501.

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for C₂H₄N₂O [M − CH₂CONHPr]+ m/z calc 227.1179, obsd 227.1181.

7-[(1-(Benzoxylimino)ethyl)-1-(4-nitrophenyl)-3-(N-propylcarbamoyl)indolizine-1-carboxylate 12. The pyridinium salt 11 (120 mg, 0.29 mmol) and para-nitrophenylpropiolic ester S (62 mg, 0.32 mmol) were dissolved in CH₂CN (3 mL). K₂CO₃ (42 mg, 0.3 mmol) was then added and the mixture was stirred overnight at rt. The solid was filtered off and the solution was concentrated under reduced pressure. The residue was then purified by flash chromatography on silica gel (cyclohexane/ethyl acetate: 7:3). Indolizine 12 was thus isolated as a yellow solid (34 mg, 23%). ¹H NMR (400 MHz, CDCl₃) δ 9.63 (d, 1H, J = 7.6 Hz), 8.44 (d, 1H, J = 1.2 Hz), 8.81 (d, 2H, J = 9.2 Hz), 7.78 (s, 1H), 7.57 (dd, 1H, J = 7.6, 2.0 Hz), 7.37−7.48 (m, 7H), 6.27 (br s, 1H), 5.34 (2H, s), 4.50 (q, 2H, J = 6.8 Hz), 2.33 (s, 3H), 1.73 (m, 2H), 1.07 (t, 3H, J = 7.2 Hz). ¹³C NMR (100 MHz, CDCl₃) δ 161.3, 161.1, 155.8, 152.4, 145.1, 139.0, 137.6, 134.5, 128.5, 128.3, 128.0, 127.9, 125.3, 122.7, 71.1, 71.0, 70.9, 70.1, 40.8, 28.5; HRMS (ESI) m/z: calc for C₂₉H₂₉N₅O₇ [M + Na]+ 915.1924, obsd 915.1925 and calc for C₂₉H₂₉N₅O₇ [M−OPh⁺] 376.1656, obsd 376.1654.

N-[2-(2-(2-Tert-Butyloxy carbamolyloxyethoxy)-ethoxy)ethyl]pyridin-4-carboxamide 14. A mixture of 2-(2-(2-tert-butyloxy carbamolyloxyethoxy)ethoxy)ethylamine (407 mg, 1.64 mmol) and N,N-diisocyanotetraester 13 (400 mg, 1.64 mmol) and NEt₃ (0.22 μL, 1.64 mmol) in CH₂Cl₂ (10 mL) was stirred overnight at 30 °C. The mixture was then diluted with CH₂Cl₂ and washed twice with saturated aqueous K₂CO₃ (pH > 10) and twice with water and brine. The organic phase was stirred under microwave irradiation for 2 days. The solution was then triturated in Et₂O and then filtered. The red solid was washed with slow filtration to afford the indolizine 16 in good purity, as indicated by ¹H NMR. An analytically pure sample (25 mg) was obtained by purification on SiO₂ column with AcOEt/cyclohexene 8/2 (v/v) solvent mixture. ¹H NMR (400 MHz, CDCl₃) δ 9.52 (br s, 1H), 8.65 (br s, 1H), 8.25 (d, 2H, J = 9.2, 2.4 Hz), 8.09 (s, 1H), 7.49 (br s, 1H), 7.39 (d, 2H, J = 8.8 Hz), 7.12 (br s, 1H), 4.88 (br s, 1H), 3.70−3.50 (m, 8H), 3.47−3.45 (m, 2H), 3.20−3.17 (m, 2H), 1.32 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 161.1, 155.7, 145.3, 138.7, 133.2, 128.2, 125.2, 122.7, 117.5, 111.8, 113.7, 105.0, 70.2, 69.7, 51.9, 40.3, 40.2, 28.4; HRMS (ESI) m/z: calc for C₂₅H₂₅N₅O₇S [M + Na]+ 640.2307, obsd 640.2307; calc for C₂₅H₂₅N₅O₇S [M + H]+ 618.2481, obsd 618.2480.

Biobutynyl-Isonicotinamide 18. A suspension of NH₂EG-Biotin.TFA 17 (350 mg, 0.72 mmol), NEt₃ (138 μL, 1 mmol), and para-nitrophenyl isonicotinocetic ester 13 (199 mg, 0.8 mmol) in CH₂Cl₂ (5 mL) was vigorously stirred overnight at rt. Et₂O (10 mL) was then added, and the resulting mixture was stirred for 15 min. The white solid was filtered off, washed several times with Et₂O, and dried. Biobutynyl-isonicotinamide 18 was thus isolated as a white powder (296 mg, 62%). ¹H NMR (400 MHz, CDCl₃) δ 8.73 (dd, 2H, J = 4.8, 2.4 Hz), 7.83 (dd, 2H, J = 4.8, 2.4 Hz), 4.52 (dd, 1H, J = 7.6, 4.8 Hz), 4.33 (dd, 1H, J = 8.0, 4.8 Hz), 3.74−3.64 (m, 8H), 3.58−3.56 (t, 2H, J = 5.6 Hz), 3.38 (t, 2H, J = 5.6 Hz), 3.23 (m, 1H), 2.96 (dd, 1H, J = 12.8, 5.2 Hz), 2.73 (dd, 1H, J = 12.8 Hz), 2.23 (t, 2H, J = 7.2 Hz), 1.78−1.61 (m, 4H), 1.47 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 174.6, 166.3, 164.6, 149.5, 142.4, 121.5, 69.8, 69.7, 69.1, 68.8, 61.8, 60.1, 55.3, 39.5, 38.7, 35.2, 28.2, 28.0, 25.3; HRMS (ESI) m/z: calc for C₂₅H₂₅N₅O₇S [M + Na]+ 502.2095, obsd 502.2095; calc for C₂₅H₂₅N₅O₇S [M + H]+ 480.2275, obsd 480.2276; calc for C₂₅H₂₅N₅O₇S [M + H]+ 240.6174, obsd 240.6181.

Biobutynyl-Pyridinyl Bromide 19. Biobutynyl-isonicotinamide 18 (400 mg, 0.83 mmol) was dissolved in MeOH (15 mL). Methyl bromoacetate (0.41 mL, 4 mmol) was added, and the solution was stirred under microwave irradiation for 2 days. After evaporation of the solvent, the oily residue was stirred in acetone to eliminate the excess of reagent. The oil was separated and dried (472 mg, 0.74 mmol). The crude biobutynyl-pyridinyl bromide 19 was purified by slow crystallization from MeOH. ¹H NMR (400 MHz, CDCl₃) δ 9.13 (d, 2H, J = 6.4 Hz), 8.49 (d, 2H, J = 6.4 Hz), 5.70 (s, 1H), 4.48 (m, 1H), 4.31 (m, 1H), 3.87 (s, 3H), 3.71−3.64 (m, 8H), 3.54 (t, 2H, J = 5.6 Hz), 3.34 (t, 2H, J = 5.6 Hz), 3.20 (m, 1H), 2.92 (dd, 1H, J = 12.8, 4.8 Hz), 2.69 (d, 1H, J = 12.4 Hz), 2.21 (t, 2H, J = 7.2 Hz), 1.78−1.51 (m, 4H), 1.42 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 174.8, 166.2, 164.6, 162.8, 150.2, 147.1, 125.7, 69.9, 69.8, 69.1, 68.6, 61.9, 60.2, 55.6, 52.8, 40.0, 39.6, 38.8, 35.3, 28.3, 28.1, 25.4; HRMS (ESI) m/z: calc for C₂₂H₁₈N₃O₇S [M + Na]+ 552.2486, obsd 552.2486; calc for C₂₂H₁₈N₃O₇S [M + H]+ 240.6174, obsd 240.6180.
Biotinylated-Indolizine 20. Biotinylated-pyridinium bromide 19 (140 mg, 0.25 mmol) and para-nitrophenyl propiolate 5 (140 mg, 0.75 mmol) were dissolved in DMF (1.5 mL). K₂CO₃ (35 mg, 0.25 mmol) was then added and the solution was stirred at rt for 6 h. A large volume of Et₂O was added to the solution, and the resulting suspension was stirred for 15 min and then filtered. The red solid was stirred in CH₂Cl₂ and filtered. The organic phase was evaporated to dryness and used in the step without further purification. ¹H NMR (400 MHz, CDCl₃) δ 9.49 (d, 1H, J = 7.2 Hz), 8.70 (br s, 1H), 8.25 (d, 2H, J = 9.2 Hz), 8.08 (s, 1H), 7.79 (br s, 1H), 7.53 (dd, 1H, J = 7.2, 2.0 Hz), 7.38 (d, 2H, J = 9.2 Hz), 6.44 (br s, 1H), 6.18 (s, 1H), 5.32 (s, 1H), 4.37 (m, 1H), 4.18 (m, 1H), 3.90 (s, 3H), 3.69 – 3.51 (m, 8H), 3.49 – 3.45 (m, 2H), 3.39 – 3.35 (m, 2H), 3.10 – 2.99 (m, 1H), 2.78 (dd, 1H, J = 13.2, 5.2 Hz), 2.58 (d, 1H, J = 12.8 Hz), 2.07 (t, 1H, J = 7.6 Hz), 1.62 – 1.45 (m, 4H), 1.30 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 172.2, 164.2, 162.8, 160.1, 160.0, 154.6, 144.2, 137.7, 131.3, 127.1, 124.2, 121.7, 115.3, 112.9, 103.8, 69.1, 68.8, 68.7, 60.7, 59.1, 54.3, 52.4, 50.9, 39.4, 39.2, 38.0, 34.8, 26.9, 24.4; HRMS (ESI) m/z: calcd for C₅₀H₇₀N₁₀O₁₀S₂ [M + Na]+ 1057.4610, obsd 1057.4611;

Biotinylated-COB223. Biotinylated-indolizine 20 was dissolved in CH₂Cl₂ (2 mL) and N-dansyl-bis(aminopropyl)-piperazine 21a (100 mg, 0.23 mmol) was added and the solution was stirred 72 h at rt. After evaporation of the solvent, the residue was dissolved in the minimum amount of CH₂Cl₂ and chromatographed onto SiO₂ short column with AcOEt/MeOH 5/5 to 3/7 (v/v) solvent gradient. The desired compound corresponded to a fl flitered. The red solid was stirred in CH₂Cl₂ and solved in CH₂Cl₂ (2 mL), and was stirred at rt for 6 h. A large volume of Et₂O was added to the resulting salt that was obtained as an oil in 44% yield. ¹H NMR (400 MHz, CD₃OD) δ 8.11 (d, 1H, J = 7.8 Hz), 7.63 (d, 1H, J = 5.0 Hz), 7.45 (dd, 1H, J = 7.4, 0.9 Hz), 6.93 (br s, 1H), 6.80 (d, 1H, J = 7.4 Hz), 6.76 (d, 1H, J = 7.4 Hz), 6.66 (d, 1H, J = 7.4 Hz), 4.37 (br s, 1H), 4.07 (br s, 1H), 3.98 (s, 3H), 3.79 – 3.65 (m, 12H), 3.64 – 3.56 (m, 4H), 3.57 (s, 6H), 3.45 (m, 2H), 3.38 (m, 2H), 3.22 (m, 1H), 3.09 (m, 1H), 2.96 (dd, 1H, J = 12.9, 5.0 Hz), 2.78 (d, 1H, J = 12.8 Hz), 2.20 (m, 4H), 2.04 (m, 2H), 1.77 – 1.67 (m, 1H), 1.66 – 1.50 (m, 3H), 1.45 – 1.35 (m, 2H); HRMS (ESI) m/z: calcd for C₄₅H₆₅N₉O₉S₂ [M + Na]+ 1535.4795; obsd 1535.4736.

Biological Affinity Chromatography of COB223/236-Binding Proteins from Human Placenta. Samples (0.2 mg) of biotinylated-COB223 or biotinylated-COB236 were separately bound onto two 2 mL columns of strepavidin-agarose for 20 min at room temperature. The columns were then equilibrated in Tris 20 mM-HCl buffer pH 7.5. Human placental tissue was homogenized in Tris 20 mM-HCl buffer pH 7.5 supplemented with 0.15 M NaCl and 0.1% Triton X-100, and the cytosolic extracts were prepared by centrifugation at 20 000 g. Fractions of 10 mL of placental cytosolic extracts (containing 35 mg of proteins) were diluted twice in Tris 20 mM-HCl buffer pH 7.5 and loaded onto each affinity column, which was then kept at 4 °C. The filtrates were collected and the columns were sequentially washed with 10 mL of Tris buffer 0 M NaCl and 6 mL Tris buffer before elution using 0.2 M NaCl, 6 mL Tris buffer, 0.5 M NaCl, and 6 mL Tris buffer and 1 M NaCl. All of the steps were performed at 4 °C in the presence of protease inhibitors (Sigma). Proteins eluted with 0.2 M NaCl and 0.5 M NaCl were submitted to mass spectrometry-based analyses.

Mass Spectrometry-Based Proteomic Analyses. Protein preparation and mass spectrometry-based proteomic analyses were realized as previously described. ¹Briefly, extracted proteins were stacked in the top of a SDS-PAGE gel (NuPAGE 4–12%, Invitrogen) before in-gel digestion using trypsin (Promega, sequencing grade). Resulting peptides were analyzed by online nanoLC–MS/MS (Ultimate 3000 and LTQ-Orbitrap Velos Pro, Thermo Scientific) using a 120-min gradient. Peptides were identified through concomitant searches against the Uniprot databank (Homo sapiens taxonomy) and a classical contaminants database (in-house) and the corresponding reversed databases using Mascot (version 2.5). The Proline software (http://proline.profilproteomes.fr) was used to filter the results (conservation of rank 1 peptides, peptide identification FDR <1% as calculated on peptide scores by employing the reverse database strategy, minimum peptide score of 25, and minimum of 1 specific peptide per identified protein group) before performing a compilation, grouping, and comparison of the protein groups from the COB223 and COB236 samples. Proteins from the contaminants database and additional kinetins were discarded from the final list of identified proteins. Proteins were considered as enriched in COB223 samples if they were identified only in these samples with a minimum of three weighted spectral counts or enriched at least five times in COB223 samples compared to COB236 ones.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01184.

Chemical stability of the COB-biotin modified beads, NMR spectra and HPLC chromatograms, affinity chromatography and mass spectrometry-based proteomic analysis (PDF)

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REFERENCES
(1) Goldmann, A. S.; Glassner, M.; Inglis, A. J.; Barner-Kowollik, C. Post-Functionalization of Polymers via Orthogonal Ligation Chemistry. Macromol. Rapid Commun. 2013, 34, 810–849.
(2) McKay, C. S.; Finn, M. G. Click Chemistry in Complex Mixtures: Bioorthogonal Bioconjugation. Chem. Biol. 2014, 21, 1075–1101.
(3) Beal, D. M.; Albrow, V. E.; Burslem, G.; Hitchen, L.; Fernandes, C.; Laphrott, C.; Roberts, L. R.; Selby, M. D.; Jones, L. H. Click-enabled heterofunctional template for sequential bioconjugations. Org. Biomol. Chem. 2012, 10, 548–554.
(4) Beal, D. M.; Jones, L. H. Molecular Scaffolds Using Multiple Orthogonal Conjugations: Applications in Chemical Biology and Drug Discovery. Angew. Chem., Int. Ed. 2012, 51, 6320–6326.
(5) Clavé, G.; Volland, H.; Flender, M.; Gasparotto, D.; Romieu, A.; Renard, P.-Y. A universal and ready-to-use heterofunctional cross-linking reagent for facile synthetic access to sophisticated bioconjugates. Org. Biomol. Chem. 2010, 8, 4329–4345.
(6) Reddy, R. E.; Chen, Y.-Y.; Johnson, D. D.; Beligere, G. S.; Rege, S. D.; Pan, Y.; Thottathil, J. K. An efficient synthesis of a heterobifunctional coupling agent. Bioconjugate Chem. 2005, 16, 1323–1328.
(7) Vanel, V.; Picha, J.; Fabre, B.; Budzienkow, M.; Lepšík, M.; Jiruček, J. The development of a versatile trifunctional scaffold for biological applications. Eur. J. Org. Chem. 2015, 2015, 3689–3701.
(8) Engelen, M.; Lombardi, A.; Vitale, R.; Lista, L.; Maglio, O.; Pavone, V.; Nasti, F. Branched porphyrins as functional scaffolds for multisite bioconjugation. Biotechnol. Appl. Biochem. 2015, 62, 383–392.
(9) Baker, M. B.; Ghiviriga, I.; Castellano, R. K. Molecular multifunctionalization via electronically coupled lactones. Chem. Sci. 2012, 3, 1095–1099.
(10) Viala, G.; Dautrey, S.; Maindron, N.; Hardouin, J.; Renard, P.-Y.; Romieu, A. The first "ready-to-use" benzene-based heterofunctional cross-linker for multiple bioconjugation. Org. Biomol. Chem. 2013, 11, 2693–2705.
(11) Bonte, S.; Ghinea, I. O.; Dinica, R.; Baussanne, I.; Demeunynck, M. Investigation of the Pyridinium Cation—Alkylene CyloadDITION as a Fluorogenic Coupling Reaction. Molecules 2016, 21, 332.
(12) Singh, G. S.; Mnatle, E. Recent progress in synthesis and bioactivity studies of indolines. Eur. J. Med. Chem. 2011, 46, 5237–5257.
(13) Hodgkiss, R. J.; Middleton, R. W.; Parrick, J.; Rami, H. K.; Wardman, P.; Wilson, G. D. Bioreductive fluorescent markers for hypoxic cells: a study of 2-nitroimidazoles with 1-substituents containing fluorescent, bridgehead-nitrogen, bicyclic systems. J. Med. Chem. 1992, 35, 1920–1926.
(14) Kim, E.; Lee, Y.; Lee, S.; Park, S. B. Discovery, Understanding, and Bioapplication of Organic Fluorophore: A Case Study with an Indolizine-Based Novel Fluorophore, Seoul-Fluor. Acc. Chem. Res. 2015, 48, 538–547.
(15) Marangoci, N.-L.; Popovici, L.; Ursu, E.-L.; Danac, R.; Clima, L.; Cojocaru, C.; Coroaba, A.; Neamtu, A.; Mangalagiu, I.; Pintea, M.; Rotaru, A. Pyridyl-indolizine derivatives as DNA binders and pH-sensitive fluorescent dyes. Tetrahedron 2016, 72, 8215–8222.
(16) Delattre, F.; Woisel, P.; Bria, M.; Surpateanu, G. Structural investigations of pyridin-4-yl indolizine modified β-cyclodextrin derivatives as fluorescent chemosensors for organic guest molecules. Carbohydr. Res. 2005, 340, 1706–1713.
(17) Becue, M.; Landy, D.; Delattre, F.; Cazier, F.; Fourmentin, M. Fluorescent Indolizin-b-Cyclodextrin Derivatives for the Detection of Volatile Organic Compounds. Sensors 2008, 8, 3689.
(18) Wu, X.; Cao, H.; Li, B.; Yin, G. The synthesis and fluorescence quenching properties of well soluble hybrid graphene material covalently functionalized with indolizine. Nanotechnology 2011, 22, No. 075202.
(19) Desroches-Castan, A.; Quelard, D.; Demeunynck, M.; Constant, J.-P.; Dong, C.; Keramidas, M.; Coll, J.-L.; Barette, C.; Lafanechère, L.; Feige, J.-J. A new chemical inhibitor of angiogenesis and tumorigenesis that targets the VEGF signaling pathway upstream of Ras. Oncotarget 2015, 6, 5382.
(20) Bonte, S.; Ghinea, I. O.; Baussanne, I.; Xuereb, J.-P.; Dinica, R.; Demeunynck, M. Investigation of the lipase-catalyzed reaction of aliphatic amines with ethyl propiolate as a route to N-substituted propioamide. Tetrahedron 2013, 69, 5495–5500.
(21) Delattre, F.; Woisel, P.; Surpateanu, G.; Bria, M.; Cazier, F.; Decock, P. 1, 3-Dipolar cycloaddition reaction of bipyrindayl ylides with the propynamido-β-cyclodextrin. A regiospecific synthesis of a new class of fluorescent β-cyclodextrins. Tetrahedron 2004, 60, 1557–1562.
(22) Christensen, J. B. A simple method for synthesis of active esters of isonicotinic and picolinic acids. Molecules 2001, 6, 47–51.
(23) Tokuhisa, H.; Liu, J.; Omori, K.; Kanasato, M.; Hiratani, K.; Baker, L. A. Efficient biosensor interfaces based on space-controlled self-assembled monolayers. Langmuir 2009, 25, 1633–1637.
(24) Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 1995, 57, 289–300.
(25) Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 2008, 4, 44–57.
(26) Rhee, S. Y.; Wood, V.; Dolinski, K.; Draghici, S. Use and misuse of the gene ontology annotations. Nat. Rev. Genet. 2008, 9, 509–515.
(27) Zhang, Y.; Linn, D.; Liu, Z.; Melamed, J.; Tavora, F.; Young, C. Y.; Burger, A. M.; Hamburger, A. W. EB1P, an ErB3-binding protein, is decreased in prostate cancer and implicated in hormone resistance. Mol. Cancer Ther. 2008, 7, 3176–3186.
(28) Zhang, Y.; Akinnade, D.; Hamburger, A. W. Inhibition of heregulin mediated MCF-7 breast cancer cell growth by the ErB3 binding protein EB1P. Cancer Lett. 2008, 265, 298–306.
(29) Troll, T.; Beckel, H.; Lentner-Böhm, C. Chemical electrochemistry of substituted indolines; UV and fluorescence spectra. Tetrahedron 1997, 53, 81–90.
(30) Mahon, J.; Mehta, L.; Middleton, R.; Parrick, J; Rami, H. Studies of some indolizine derivatives useful as fluorophores. J. Chem. Res. Symp. 1992, 11, 362–363.
(31) Wright, M.; Sieber, S. Chemical proteomics approaches for identifying the cellular targets of natural products. Nat. Prod. Rep. 2016, 33, 681–708.
(32) Simons, M.; Gordon, E.; Claesson-Welsh, L. Mechanisms and regulation of endothelial VEGF receptor signaling. Nat. Rev. Mol. Cell Biol. 2016, 17, 611–625.
(33) Delattre, F.; Woisel, P.; Surpateanu, G.; Cazier, F.; Blach, P. 1-(4-Nitrophenoxyacarbonyl)-7-pyridin-4-yl indolizine: A new versatile fluorescent building block. Application to the synthesis of a series of fluorescent β-cyclodextrins. Tetrahedron 2005, 61, 3939–3945.
(34) Reizelmann, A.; Wigchert, S. C. M.; del-Bianco, C.; Zwanenburg, B. Synthesis and bioactivity of labelled germination stimulants for the...
isolation and identification of the strigolactone receptor. Org. Biomol. Chem. 2003, 1, 950–959.

(35) Casabona, M. G.; Vandenbrouck, Y.; Attree, I.; Couté, Y. Proteomic characterization of Pseudomonas aeruginosa PAO1 inner membrane. Proteomics 2013, 13, 2419–2423.