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Graphical Abstract

Epoxides related to dioncoquinone B: Synthesis, activity against multiple myeloma cells, and search for the target protein

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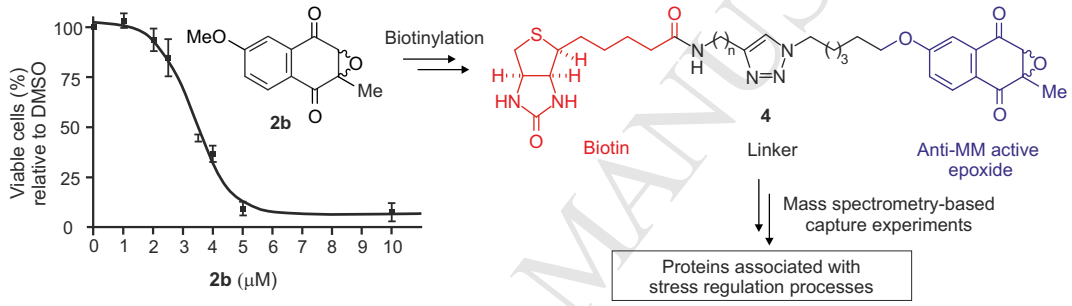

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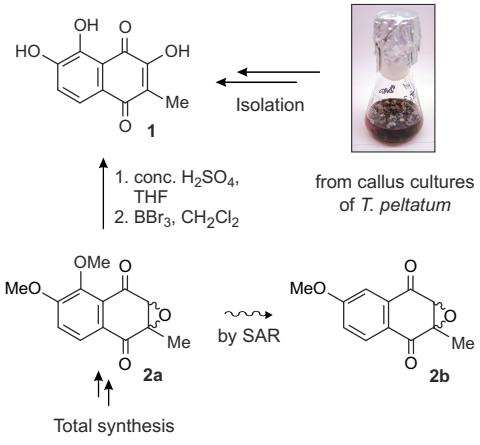
ABSTRACT

Epoxide **2b** is an analog of the synthetic intermediate **2a** en route to the polyketide-derived antitumoral naphthoquinone dioncoquinone B (**1**), isolated from cell cultures of the tropical liana *Triphyophyllum peltatum* (Dioncophyllaceae). Compound **2b** was found to induce strong apoptosis in multiple myeloma cells at a concentration (EC_{50} = 3.5 µM), distinctly lower than that of **1** and any related analog, without exerting significant toxicity against normal blood cells. Preliminary studies showed that **2b** follows different SAR rules as compared to the naphthoquinones. Among the series of synthesized epoxides, **2b** was the most active one and was thus, after biotinylation, subjected to mass spectrometry-based affinity capture experiments aiming at the identification of target proteins. The MS data revealed **2b** to address proteins that are associated with stress regulation processes which are critical for multiple myeloma cell survival.

1. Introduction

Multiple myeloma (MM), the second most prevalent hematological malignancy, is caused by an excess of abnormal plasma cells forming tumors in multiple locations within the bone marrow, thus leading to osteolytic bone destruction, impaired hematopoiesis and renal failure.[1-4] Several novel therapies have substantially improved patient outcomes over the past decade. However, with a five-year survival of only 49%,[5] cure still remains elusive. In particular, once MM patients become refractory to the major classes of anti-MM agents (proteasome inhibitors, immunomodulatory drugs, and antibodies against SLAMF7 and CD38), their median overall survival is less than twelve months.[6-9] Therefore, therapy-refractory patients have the most urgent unmet clinical need for novel therapeutic approaches.

Solid callus cultures of the tropical liana *Triphyophyllum peltatum* (Dioncophyllaceae) have previously been found to produce structurally unique polyketide-derived naphthylisoquinoline alkaloids along with a series of highly oxygenated, previously unknown naphthoquinones, among them dioncoquinone B (**1**) (Scheme 1).[10,11] This naphthoquinone is structurally related to the naphthalene part of the alkaloids. To study more closely the tumor-specific activities of **1** and related naphthoquinones, malignant cells of the human MM cell line INA-6[10-14] have been used during that work, which showed that dioncoquinone B (**1**) exerts a pronounced cytotoxicity towards MM cells (EC_{50} = 11 µM), without any noticeable toxic effects against normal peripheral mononuclear blood cells (PBMCs).[10] For more in-depth investigations on the anti-MM potential of those naphthoquinones, a synthetic pathway (Scheme 1), affording **1** in larger quantities and simultaneously providing a small library of related compounds, has been developed.[11]



Scheme 1. Preparation of dioncoquinone B (**1**) by isolation and by total synthesis via the epoxide **2a**, and structure of its optimized analog **2b**.

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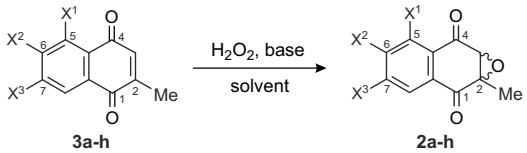
Table 1. Synthesis of epoxides **2a-h** from their respective naphthoquinone precursors **3a-h** under basic conditions.



3a-h **2a-h**

Entry	Starting material	Product	X^1	X^2	X^3	Reaction conditions	Yield (%)
1	**3a**[11]	**2a**[11]	OMe	OMe	H	K_2CO_3, THF	97
2	**3b**	**2b**[11]	H	OMe	H	K_2CO_3, THF	99
3	**3c**[25]	**2c**[24]	H	Me	H	NaOH, MeOH/H_2O	93
4	**3d**[11]	**2d**[11]	H	OH	H	K_2CO_3, THF	92
5	**3e**	**2e**	H	CO_2Me	H	NaOH, MeOH/H_2O	72
6	**3f**[11]	**2f**[11]	H	OMe	OMe	K_2CO_3, THF	87
7	**3g**[11]	**2g**[11]	OMe	OMe	OMe	NaOH, MeOH/H_2O	99
8	**3h**[24]	**2h**	OMe	Cl	H	NaOH, MeOH/H_2O	71

In the course of these investigations, we also tested the anti-MM potential of selected synthetic precursors, among them the intermediate epoxide **2a**, which can easily be prepared from the respective naphthoquinone precursor and can then be converted into the target molecule **1** under acidic conditions (Scheme 1). These investigations revealed that **2a** and some related epoxides of the general structure **2** showed a distinctly better activity than the naphthoquinone **1**, combined with a significantly reduced cytotoxicity.

In this paper, we report on the synthesis of **2a** and on the SAR-guided preparation of a whole series of related epoxides of type **2**. Among them, the oxygen-poorer analog **2b** was found to induce strong apoptosis in MM cell lines at a concentration (3.5 µM), lower than that of any of the tested dioncoquinones and their related synthetic analogs.[10,11]

We further describe the labeling of **2b** by biotinylation for the identification of the target protein and ensuing mass spectrometry-based capture experiments aiming at the identification of the target proteins.

2. Results and discussion

2.1. Synthesis and biotesting of the epoxides

The synthesis of the required naphthoquinones **3a**, **3c**, **3d**, **3f-3h** was done according to known experimental procedures[11,24,25] or, in the case of **3b** and **3e**, to analogous protocols, following in principle three different approaches, either by a Stobbe strategy, via a Grignard route, or by a Diels-Alder pathway.

Likewise in analogy to previous protocols,[11,24] epoxidation of the naphthoquinones **3a-h** succeeded by using hydrogen peroxide under different optimized conditions, nearly all of the compounds were obtained in very good yields and purity (Table 1).

The epoxides were tested for their growth-retarding activity against MM cells according to a protocol established earlier,[10-14] by determination of the viability of INA-6 cells 3 d after treatment. The results (Table 2) showed that all of the epoxides (except for compound **2d**, which was the only one with an EC_{50} value of > 10 µM) exhibited significant antitumoral activities against INA-6 cells in a low micromolar range, similar to that of the DNA-alkylating agent melphalan, which is utilized in the standard therapy of MM.[26,27] Epoxide **2e** possessing a carboxylate function at C-6, was found to be the most potent compound (EC_{50} = 0.6 µM) – yet combined with an even stronger cytotoxicity against the non-malignant PMBCs (EC_{50} = 0.4 µM). The overall best anti-MM activity was displayed by **2b**, with an EC_{50} value of 3.5 µM, while lacking any cytotoxic effects in normal PMBCs.

In total, the structure-activity relationship (SAR) studies showed that for a good anti-MM activity (and low cytotoxicity), C-5 and C-7 should be unsubstituted, and C-6 should bear an alkoxy group, and there should be no free hydroxy functions. This SAR profile clearly distinguished the epoxide of the general structure **2** from naphthoquinones of type **1**, where we had previously found that there should be free OH groups at C-3, C-5, and/or C-6, evidencing that such epoxides **2** follow a clearly different mode of action as compared to the previously described naphthoquinones.

Table 2. EC_{50} values (µM) of INA-6 multiple myeloma cells and peripheral mononuclear blood cells (PMBCs) treated with epoxides **2a-h**.

Entry	Compound	EC_{50} (µM)	
		INA-6	PBMC
1	**2a**	2.8	7.4
2	**2b**	3.5	NR
3	**2c**	2.0	2.0
4	**2d**	13.0	37.0
5	**2e**	0.6	0.4
6	**2f**	5.0	11.0
7	**2g**	2.2	3.8
8	**2h**	3.0	4.5
9	**1**	11.0	NR
10	melphalan	2	3

NR: not reached.

[a] Activity against the multiple myeloma cell line INA-6.

[b] Cytotoxicity against normal peripheral mononuclear blood cells (PBMC).

*2.2. Biotinylation of the epoxide **2b***

The high anti-MM activity of the epoxides, in particular of **2b**, made it rewarding to launch first investigations on the mode of action.

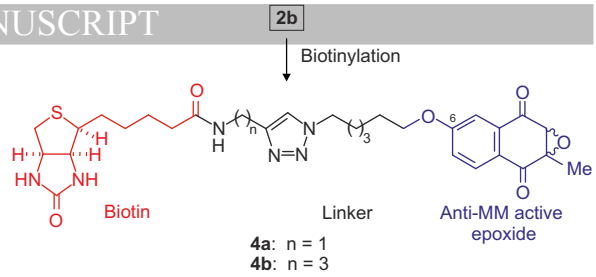
Despite the growing number of successful examples, identification of molecular targets still remains a complex challenge.[28-30] One of the most powerful and frequently used methods is the derivatization of a bioactive molecule to a pulldown probe to determine the cellular binding partners by a protein affinity isolation combined with mass-spectrometric analysis of the captured proteins. Biotin as such an efficient affinity tag, which enables a target enrichment utilizing the strong non-covalent interaction with streptavidin,[29,31] was chosen for the labeling of the anti-MM active epoxide **2b**.

The design of a functionalized derivative of **2b** (Scheme 2) was planned to be based on the attachment of biotin to the known[11] epoxide **2d** through a triazol linker, by applying the Cu(I)-catalyzed Huisgen reaction (CuAAC, 'click reaction').[32-35] This mild method seemed to be well suited in order to avoid any ring opening reaction of the epoxide function of **2d**, although epoxides of type **2** had previously been found to be relatively stable towards nucleophiles.[36]

As the site for the attachment of a linker-tethered biotin molecule, the oxygen function at C-6 was addressed because the above-described structure-activity studies had shown that this position should bear an alkoxy function, as given in the tethered compound **4**. This seemed better than any derivatization at C-5 or C-7. A molecule of type **4** should also permit easy variation of the chain length within the central linker part.

As starting material for the click reaction, the biotin residue was chosen to contain the alkyne part, while the bioactive epoxide was planned to constitute the azide part. The preparation of the known alkyne **7a** (Scheme 3) was achieved according to a established procedure from literature.[37] The synthesis of the new analog **7b**, bearing a longer chain, followed the same protocol.

Desymmetrization of the commercially available 1,6-dibromohexane (**8**) by reaction with the phenol function of **2d** led

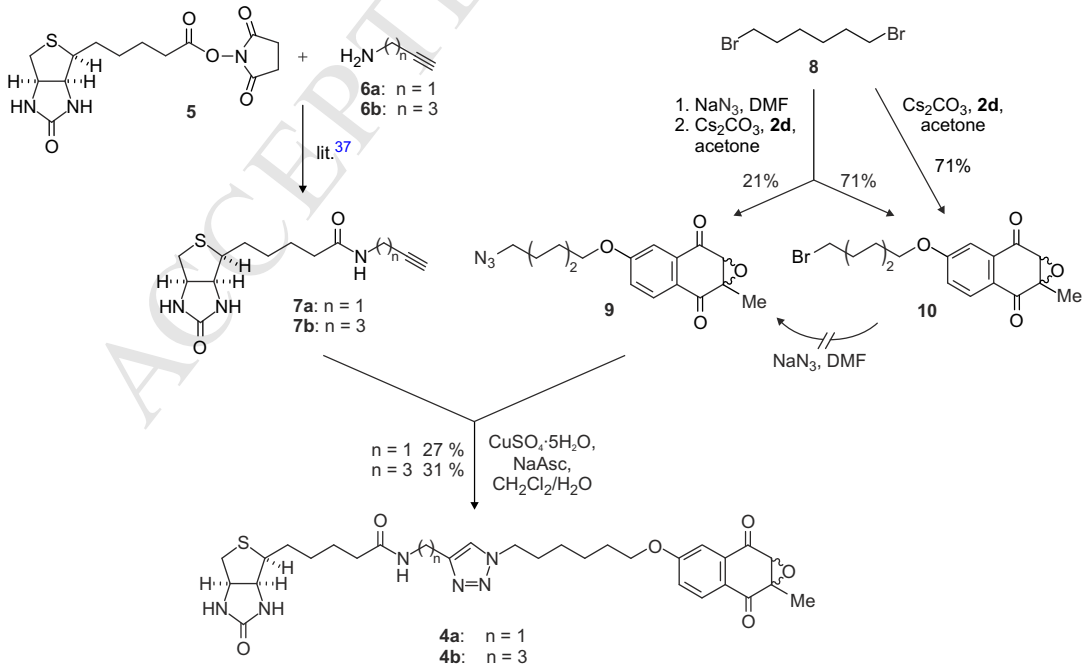


Scheme 2. General approach to a biotinylated derivative of the epoxide **2d**.

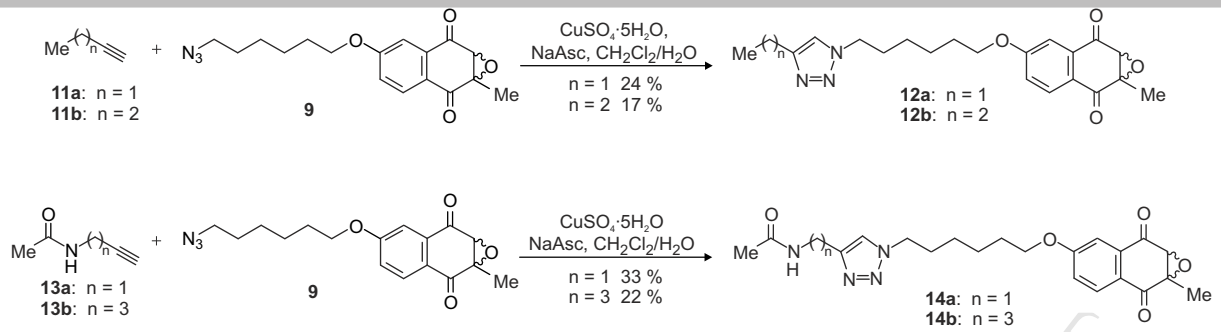
to the monoether **10** in acceptable yields (Scheme 3), but the following substitution of the remaining bromine by inorganic azide unfortunately resulted in a ring cleavage of the epoxide function. For this reason, the dibromide **8** was first substituted with azide to give a mixture of the desired 1-azido-6-bromo-hexane along with unreacted **8**. This mixture was directly submitted to the etherification of **2d**, which succeeded without ring cleavage reaction, leading to the mixture of the desired azido-functionalized epoxide **9** and the undesired bromide **10**. Despite the unsatisfying yield thus overall obtained, this sequence was acceptable due to the small quantity of labeled compound **4** finally needed. The copper(I)-catalyzed [3+2] cycloaddition of **9** with the different biotin alkynes **7a** and **7b** gave the functionalized epoxides **4a** and **4b**, respectively (Scheme 3).

2.3. Synthesis of shorter, less functionalized analogs of 4

In addition to the biotinylated analogs **4a** and **4b**, we also prepared biotin-free, less functionalized derivatives, to make sure that the linker had no influence on the anti-MM activity. Because of the increased chain length, with a simultaneously larger number of functional groups in **4** as compared to the epoxide **2b**, analogs of types **12** and **14** were synthesized (Scheme 4). The



Scheme 3. Synthesis of the azido-functionalized epoxide **9** and its click reaction with the biotin alkynes **7a** and **7b** to give the target molecules **4a** and **4b**, respectively.



Scheme 4. Analogous synthesis of biotin-free derivatives of **2b**.

commercially available alkynes **11a** and **11b** and the likewise known[38] alkynyl acetamides **13a** and **13b** were submitted to click reactions with **10** applying the same conditions as for the preparation of the target molecules **4**, leading to similar yields.

2.4. Antitumoral activities and drug target identification

In contrast to the highly active epoxide **2b**, its biotinylated analogs **4a** and **4b** showed no anti-MM activity anymore (Table 3), probably as a consequence of decreased cell permeability due to the large lipophilicity or the increased molecular size.[29,31] This assumption was confirmed by the already decreased activity of the synthesized smaller, biotin-free analogs **12** and **14** (Figure 1). The azido and bromo derivatives **9** and **10** showed good anti-MM activity, with EC_{50} values of 3.3 µM and 7.2 µM respectively, similar to that of the epoxide **2b**, yet accompanied by an increased cytotoxicity towards PMBCs. The more lipophilic derivatives **12a** and **12b**, by contrast, exerted their anti-MM activities at higher concentrations only. The closer analogs **14a** and **14b**, with only biotin missing, and the labeled compounds **4a** and **4b** themselves did not even reach measurable EC_{50} values in the whole-cell assay.

Table 3. EC_{50} values (µM) of INA-6 multiple myeloma cells and peripheral mononuclear blood cells (PMBCs) treated with the biotinylated derivatives **4a**/**b**, or with their biotin-free derivatives **9**, **10**, **12a/b**, and **14a/b**.

Entry	Compound	EC_{50} (µM)	
		INA-6	PBMC
1	**2b**	3.5	NR
2	**9**	3.3	5.5
3	**10**	7.2	6.8
4	**12a**	16.2	NR
5	**12b**	25.9	NR
6	**14a**	NR	NR
7	**14b**	NR	NR
8	**4a**	NR	NR
9	**4b**	NR	NR

NR: not reached.

[a] Activity against the multiple myeloma cell line INA-6.

[b] Cytotoxicity against normal peripheral mononuclear blood cells (PBMC).

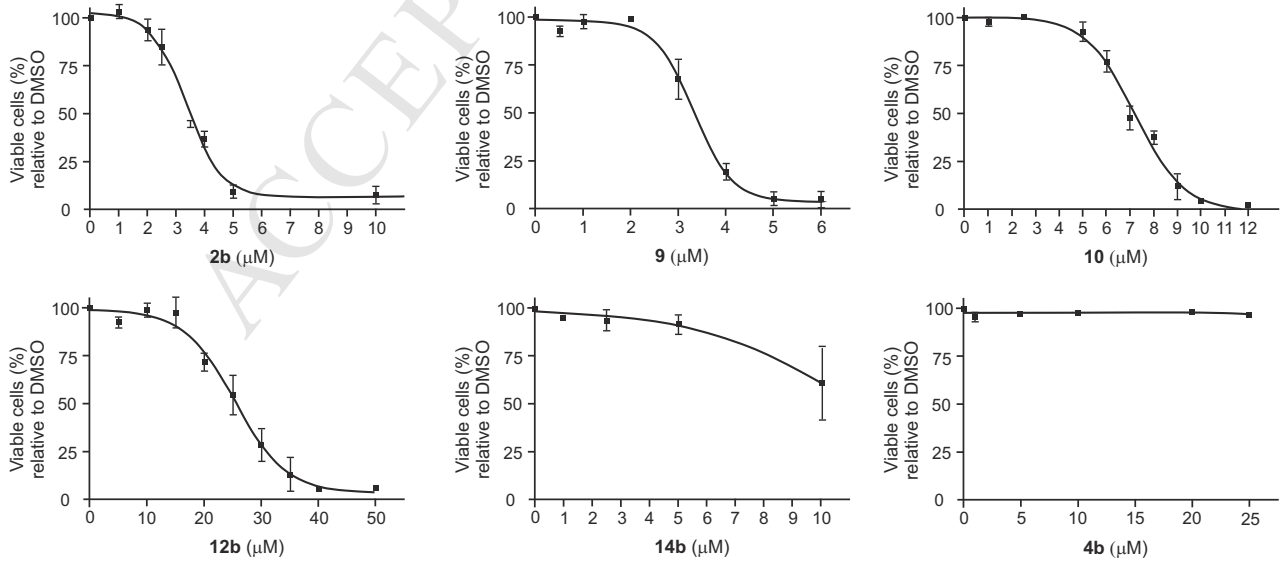


Figure 1. Anti-MM effect of the highly active epoxide **2b**, the derivatives **9**, **10**, **12b** and **14b**, and the biotinylated epoxide **4b** (data of **12a** and **14a** are not shown). INA-6 multiple myeloma cells were treated either with DMSO as the solvent control or with increasing concentrations of **2b**, **9**, **10**, **12b**, **14b**, and **4b** for 3 d. The viable fractions of the treated cells were determined by annexin V-FITC/PI staining using flow cytometry. Shown are mean values and standard deviations of three independent experiments.

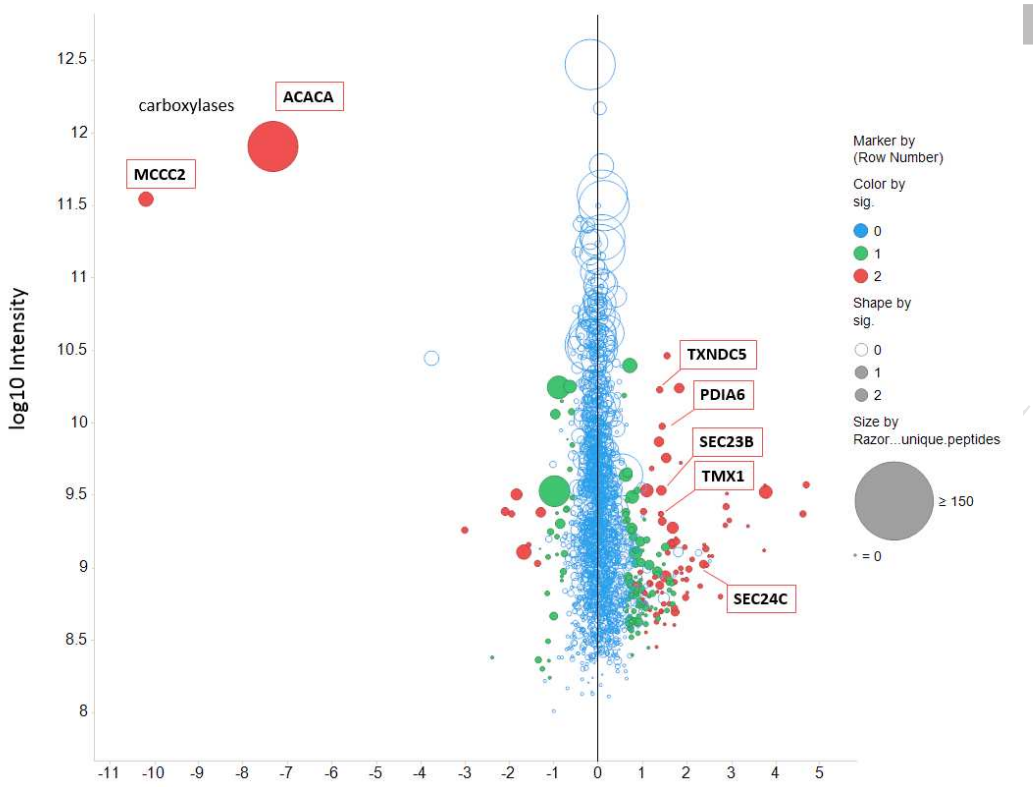


Figure 2. Identification of potential protein targets from the affinity capture experiment with **4b** and INA-6 cell lysate (for **4a** not shown). Proteins significantly enriched over the control are represented by closed circles. Outliers are shown in green, extreme outliers in red. Protein ratios and intensities are median values from three biological replicates (n replicates >1). The size of the circles correlates with the number of identified razor and unique peptides. Carboxylases were enriched specifically with the streptavidin control beads as they have biotin as the prosthetic group.

These results were not discouraging, because they only showed that the biotinylated molecules **4a** and **4b** were not active in the whole-cell test system, certainly due to the biotin residue, which should prevent the permeation through the cell membrane.[29,31] Thus, it seemed obvious that the mechanistic investigations should inevitably be performed in cell lysate.

For the target identification, the biotinylated compounds **4a** and **4b** were subjected to mass spectrometry-based affinity capture experiments. The magnetic bead preparation, cell lysis, drug target enrichment and in-solution digestion followed previously published experimental procedures.[14] Therefore, the biotinylated inhibitors **4a** and **4b** loaded on streptavidin magnetic beads and empty beads as the control were incubated with INA-6 cell lysate under the same condition (3 h at 4 °C). After washing the precipitate, the captured proteins were eluted with lithium dodecyl sulfate (LDS) sample buffer, precipitated with acetone, and digested in solution with trypsin. The proteins were analyzed by nanoLC-MS/MS and label-free quantification (LFQ). To achieve reliable results, three biological replicates of each biotin-labeled compound were investigated.

The data set showed a clear enrichment of several proteins (Figure 2). Of note, the proteins targeted by the biotinylated epoxides **4a** and **4b** were similar to each other. One of the most prominent hits was peroxiredoxin 1 **(**PRDX1), a redox-regulating protein that has been reported to eliminate reactive oxygen species (ROS) and regulate cell growth, differentiation, and apoptosis.[38] PRDX1 has been found to be overexpressed in human cancer including MM, and to functionally play a tumor-promoting role in progression and metastasis.[39-41] Thus, PRDX1 mediates anti-apoptotic effects through interactions with several ROS-dependent effectors such as ASK1 and JNK. In addition, PRDX1 can associate to key cancer transcription factors such as p53, c-Myc and NF-κB and can modulate their anti-apoptotic activity.[38] Another significantly enriched protein, thioredoxin domain-containing 5 (TXNDC5), is a recently discovered endoplasmic reticulum (ER) resident disulfide isomerase, which has been implicated in oxidative-stress associated diseases including cancer. Thus, TXNDC5 acts as a chaperon-mediating protein folding and correct formation of disulfide bonds through its thioredoxin domains.[42] Recently, TXNDC5 has been shown to act as a super-enhancer to dysregulate c-Myc expression, and thus contributing to tumor progression in MM.[43] Like TXNDC5, the protein disulfide isomerase A6 (PDIA6) acts as an ER-resident chaperon, co-regulates UPR signaling, and thus contributes to protein homeostasis.[44,45] A recent study has identified a group of protein homeostasis-relevant genes including PDIA6 which are significantly regulated in therapy-resistant MM cells underscoring the critical role of ER-stress associated processes in MM.[46] Accordingly, further ER-associated proteins were found to be enriched: i) thioredoxin-related transmembrane protein 1 (TMX1), another ER-resident PDI family member, which catalyzes protein folding and thiol-disulfide interchange reactions and which is known to be involved in ER stress regulation, and ii) Sec23B and SEC24C (Sec23 homolog B, coat complex II component and SEC24 homolog C, COPII coat complex component), both are components of the coat protein complex II (COPII) and promote formation of transport vesicles from the ER.[47] Although all these enriched proteins indicate a functional link of compound **2b** to ER stress response mechanisms, which are considered to be critical for tumor cell survival in MM, further investigation on the potential target is needed and will be reported in due course.[48]

3. Experimental section

3.1. General experimental procedures

All melting points were measured on a Reichert Kofler hot-stage microscope and are uncorrected. IR spectra were recorded on a Jasco FT/IR-410 spectrometer by the ATR technique. The signal intensity is assigned using the following abbreviations: s (strong), m (medium), w (weak). ^{1}H and ^{13}C spectra were recorded at room temperature on a Bruker Avance-400 instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C). They are internally referenced to residual solvent signals and are expressed in parts per million (ppm). All coupling constants (*J*) are reported in Hertz (Hz) and the following notations indicate the multiplicity of the signals: b (broad), s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet). MS and HRMS measurements were performed on a Finnigan MAT-8200 instrument (EI) and a Bruker Daltonics micrOTOF-mass spectrometer (ESI). Thin-layer chromatography was performed on Merck precoated silica gel aluminum plates (60 F_{254}) and visualized by exposure to ultraviolet light at 254 nm and 365 nm or by staining with a potassium permanganate solution. Column chromatography was performed using silica gel (0.063 mm, Machery-Nagel). All solvents were either purchased from commercial suppliers or purified by standard techniques. Reactions sensitive to air or moisture were carried out in flame-dried glassware under nitrogen using standard Schlenk techniques. 1-Pentyne and 1-hexyne were purchased from commercial suppliers. The syntheses of the biotin alkynes and acetamides were carried out according to published procedures.[37,38] The naphthoquinones en route to the epoxides **3a**, **3c**, **3d**, **3f-h** and the epoxides **2a**, **2c**, **2d**, **2f**, **2g** were prepared as reported in earlier papers.[11,24,25]

*3.2. Synthesis of the naphthoquinones for epoxides **3b** and **3e***

*3.2.1. 6-Methoxy-2-methyl-1,4-naphthoquinone (**3b**)*

K_2CO_3 (20.6 mg, 149 µmol, 5.0 equiv) was added to a solution of 6-hydroxy-2-methyl-1,4-naphthoquinone (5.6 mg, 29.8 µmol, 1.0 equiv) in acetone (2 mL) at 0 ºC. After stirring the mixture for 10 min, dimethyl sulfate (18.8 mg, 149 µmol, 14.1 µL, 5.0 equiv) was added and the reaction solution was stirred for 3 h. Then water was added and the mixture was neutralized with 1N aqueous HCl and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated in vacuo and the residue was resolved by column chromatography (*n*-hexane/EtOAc 10:1) to give the naphthoquinone **3b** (5.9 mg, 29.2 µmol, 98%) as a yellow solid; mp 142 °C; IR (ATR) ν_{max} 2924 (w), 2851 (w), 1661 (m), 1592 (s), 1438 (w), 1361 (m), 1305 (s), 1243 (m), 1076 (m), 1027 (m), 946 (w), 896 (w), 850 (w) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.6 Hz, 1H, H_{ar}), 7.49 (d, *J* = 2.7 Hz, 1H, H_{ar}), 7.18 (dd, *J* = 8.6, 2.7 Hz, 1H, H_{ar}), 6.80 (q, *J* = 1.5 Hz, 1H, CH), 3.94 (s, 3H, OCH_3), 2.18 (d, *J* = 1.5 Hz, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.3 (C=O), 184.8 (C=O), 164.1 (C-6), 148.7 (C-2), 135.4 (C-3), 134.5 (C_{ar}), 129.2 (C_{ar}), 125.9 (C_{ar}), 120.4 (C_{ar}), 109.4 (C_{ar}), 56.1 (OCH_3), 16.7 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int.) 202 $[M]^+$ (100), 174 $[M\text{-}CO]^+$ (22), 134 (26); HRMS (ESI) calcd for $C_{12}H_{11}O_3$ $[M+H]^+$ 203.0703, found 203.0702.

*3.2.2. Methyl-6-carboxylate-2-methyl-1,4-naphthoquinone (**3e**)*

*3.2.2.1. 6-Methyl-2-naphthoic acid (**15**)*

A mixture of 2,6-dimethylnaphthalene (416 mg, 2.66 mmol, 1.0 equiv) and $KMnO_4$ (2.10 g, 13.3 mmol, 5.0 equiv) in pyridine (35 mL) and water (12 mL) was refluxed for 2 h. Then another portion of $KMnO_4$ (840 mg, 5.32 mmol, 2.0 equiv) was added. After 0.5 h the reaction mixture was cooled to room temperature and 1N aqueous NaOH was added. The solution was filtered over Celite and the solvent was concentrated in vacuo. The crude product was purified by column chromatography (*n*-hexane/EtOAc 10:1) to give the naphthoic acid **15** (403 mg, 2.16 mmol, 81%) as a white solid; mp 225–227 °C; IR (ATR) ν_{max} 3400 (m), 2980 (w), 2916 (w), 2830 (w), 1670 (s), 1574 (m), 1475 (m), 1415 (w), 1383 (m), 1290 (m), 1244 (m), 1194 (m), 1099 (m), 958 (w), 906 (w), 877 (w), 800 (m), 764 (m), 712 (m), 637 (m) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (s, 1H, H_{ar}), 8.08 (dd, *J* = 8.7, 1.2 Hz, 1H, H_{ar}), 7.88 (d, *J* = 8.5 Hz, 1H, H_{ar}), 7.82 (d, *J* = 8.7 Hz, 1H, H_{ar}), 7.67 (s, 1H, H_{ar}), 7.40 (dd, *J* = 8.5, 1.2 Hz, 1H, H_{ar}), 2.56 (s, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4 (CO_2H); 139.0 (C_{ar}), 136.4 (C_{ar}), 132.0 (C_{ar}), 130.8 (C_{ar}), 129.5 (C_{ar}), 129.2 (C_{ar}), 127.8 (C_{ar}), 127.0 (C_{ar}), 125.7 (C_{ar}), 125.7 (C_{ar}), 22.1 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 186 $[M]^+$ (100), 169 $[M\text{-}OH]^+$ (30), 141 $[M\text{-}CO_2H]^+$ (57); HRMS (ESI) calcd for $C_{12}H_{10}O_2Na$ $[M+Na]^+$ 209.0573, found 209.0572.

*3.2.2.2. Methyl-6-methyl-2-naphthoate (**16**)*

K_2CO_3 (162 mg, 1.18 mmol, 3.0 equiv) was added to a solution of 6-methyl-2-naphthoic acid (**15**) (73.0 mg, 392 µmol, 1.0 equiv) in acetone (10 mL) at 0 °C. After stirring the mixture for 30 min, dimethyl sulfate (83.8 mg, 1.22 mmol, 63 µL, 3.1 equiv) was added and the solution was refluxed for 1 h. The reaction mixture was cooled down to room temperature and H_2O was added. After evaporation of acetone, the remaining aqueous mixture was extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated in vacuo and the residue was resolved by column chromatography (*n*-hexane/EtOAc 10:1) to give the ester **16** (66.0 mg, 330 µmol, 84%) as a colorless solid; mp 119-120 °C; IR (ATR) ν_{max} 2954 (w), 2926 (w), 2851 (w), 1707 (s), 1628 (w), 1473 (w), 1435 (m), 1336 (w), 1286 (s), 1190 (s), 1124 (m), 1093 (s), 972 (m), 897 (m), 843 (w), 810 (s), 777 (m), 750 (m), 723 (w) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H, H_{ar}), 8.02 (dd, *J* = 8.6, 1.7 Hz, 1H, H_{ar}), 7.85 (d, *J* = 8.4 Hz, 1H, H_{ar}), 7.79 (d, *J* = 8.6 Hz, 1H, H_{ar}), 7.65 (s, 1H, H_{ar}), 7.38 (dd, *J* = 8.4, 1.7 Hz, 1H, H_{ar}), 3.97 (s, 3H, OCH_3), 2.54 (s, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.6 (CO_2Me), 138.5 (C_{ar}), 135.9 (C_{ar}), 131.0 (C_{ar}), 130.9 (C_{ar}), 129.3 (C_{ar}), 129.1 (C_{ar}), 127.6 (C_{ar}), 126.9 (C_{ar}), 126.7 (C_{ar}), 125.5 (C_{ar}), 52.3 (OCH_3), 22.1 (2-CH_3) ppm; MS (EI, 70 eV): *m/z* (rel int) 200 $[M]^+$ (78), 169 $[M\text{-}OCH_3]^+$ (100), 141 (32); HRMS (ESI) calcd for $C_{13}H_{12}O_2Na$ $[M+Na]^+$ 223.0730, found 223.0730.

*3.2.2.3. Methyl -2-methyl-1,4-naphthoquinone-6-carboxylate (**3e**)*

To a mixture of H_5IO_6 (299 mg, 1.31 mmol, 4.0 equiv) and CrO_3 (3.3 mg, 33.0 µmol, 0.1 equiv) in MeCN (7 mL), **16** (66.0 mg, 330 µmol, 1.0 equiv) in MeCN (7 mL) was added dropwise. After stirring at 0 ºC for 4 h, the reaction mixture was filtered over a short pad of Celite and washed with CH_2Cl_2. The solvent was concentrated in vacuo and the residue was resolved by column chromatography (*n*-hexane/EtOAc 97:3) to give the naphthoquinone **3e** (30.0 mg, 130 µmol, 40%) as a yellow solid; mp149-152 °C; IR (ATR) ν_{max} 2950 (w), 2929 (w), 2852 (w), 1716 (s), 1660 (s), 1603 (m), 1439 (m), 1356 (w), 1259 (s), 1120 (m), 972 (m), 939 (m), 912 (m), 874 (m), 766 (m), 688 (m) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (dd, *J* = 1.7, 0.5 Hz, 1H, H_{ar}), 8.37 (dd, *J* = 8.1, 1.7 Hz, 1H, H_{ar}), 8.18 (dd, *J* = 8.1 Hz, 0.5 Hz, 1H, H_{ar}), 6.91 (q, *J* = 1.6 Hz, 1H, H_{ar}), 3.98 (s, 3H, OCH_3), 2.22 (d, *J* = 1.6 Hz, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.1 (C=O), 184.2 (C=O), 165.6 (CO_2Me), 148.6 (C_{ar}), 136.2

(C_{ar}), 134.9 (C_{ar}), 134.8 (C_{ar}), 134.3 (C_{ar}), 132.4 (C_{ar}), 127.7 (C_{ar}), 127.0 (C_{ar}), 52.9 (OCH_3), 16.7 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 230 $[M]^+$ (100), 199 $[M\text{-}OCH_3]^+$ (95), 171 (47); HRMS (ESI) calcd for $C_{13}H_{11}O_4$ $[M+H]^+$ 231.0652, found 231.0645.

3.3. General procedure for the epoxidation of the naphthoquinones

3.3.1. Epoxidation with H_2O_2 and K_2CO_3 in THF

Aqueous H_2O_2 (30%, 90 equiv) was added with stirring to a mixture of the naphthoquinone (1.0 equiv) in THF (25 µL µmol^{-1}) at room temperature. After addition of 1N aqueous Na_2CO_3 until the mixture had turned colorless, the reaction solution was stirred for 3 h at room temperature. Saturated aqueous NaCl was added and the mixture was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo.

3.3.2. Epoxidation by H_2O_2 and NaOH in MeOH/H_2O

To a mixture of naphthoquinone (1.0 equiv) in H_2O/MeOH (1:4, 50 µL/µmol) 1N aqueous NaOH solution (0.5 equiv) was added at room temperature. After the mixture was stirred for 10 minutes, aqueous H_2O_2 solution (30%, 1.5 equiv) was added and stirred for 3 h. The mixture was neutralized with 1N aqueous HCl and extracted with Et_2O. The combined organic layers were dried over Na_2SO_4, and the solvent was removed in vacuo.

*3.3.3. 6-Methoxy-2-methyl-1,4-naphthoquinone-2,3-epoxide (**2b**)*

According to the general procedure 3.3.1., compound **2b** was synthesized starting from the naphthoquinone **3b** (6.0 mg, 29.7 µmol, 1.0 equiv). Purification by column chromatography (*n*-hexane/EtOAc 10:1) gave the epoxide **2b** (6.4 mg, 29.4 µmol, 99%) as yellow solid; mp 89 °C; IR (ATR) ν_{max} 1684 (s), 1592 (s), 1494 (m), 1433 (m), 1294 (s), 1232 (s), 1192 (m), 1057 (m), 1026 (m), 948 (m), 856 (s), 780 (m), 740 (s) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, J = 8.7 Hz, 1H, H_{ar}), 7.38 (d, J = 2.7 Hz, 1H, H_{ar}), 7.22 (dd, J = 8.7, 2.7 Hz, 1H, H_{ar}), 3.92 (s, 3H, OCH_3), 3.84 (s, 1H, CH), 1.72 (s, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0 (C=O), 190.8 (C=O), 164.7 (C-6), 134.2 (C_{ar}), 130.1 (C_{ar}), 125.4 (C_{ar}), 121.7 (C_{ar}), 109.9 (C_{ar}), 61.5 (C-2), 61.4 (C-3), 56.1 (OCH_3), 14.9 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 218 $[M]^+$ (100), 203 $[M\text{-}CH_3]^+$ (82), 119 (51); HRMS (ESI) calcd for $C_{12}H_{10}NaO_4$ $[M+Na]^+$ 241.0471, found 241.0470.

*3.3.4. Methyl-2-methyl-1,4-naphthoquinone-2,3-epoxide-6-carboxylate (**2e**)*

According to the general procedure 3.3.2., compound **2e** was synthesized starting from the naphthoquinone **3e** (62.0 mg, 269 µmol, 1.0 equiv). The residue was purified by column chromatography (*n*-hexane/EtOAc 9:1) to give the epoxide **2e** (48.0 mg, 193 µmol, 72%) as a yellow solid; mp 135 °C; IR (ATR) ν_{max} 2954 (w), 2929 (w), 1716 (s), 1691 (s), 1606 (m), 1444 (m), 1400 (w), 1338 (w), 1263 (s), 1184 (m), 1122 (m), 974 (w), 947 (m), 876 (w), 850 (w), 795 (w), 759 (m), 714 (s), 640 (w) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, J = 1.7 Hz, 1H, H_{ar}), 8.36 (dd, J = 8.1, 1.7 Hz, 1H, H_{ar}), 8.07 (d, J = 8.1 Hz, 1H, H_{ar}), 3.97 (s, 3H, OCH_3), 3.90 (s, 1H, CH), 1.74 (s, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6 (C=O), 191.1 (C=O), 165.2 (CO_2Me), 135.6 (C_{ar}), 135.2 (C_{ar}), 135.0 (C_{ar}), 132.2 (C_{ar}), 128.3 (C_{ar}), 127.97 (C_{ar}), 61.9 (C-2), 61.6 (C-3), 53.0 (OCH_3), 14.7 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 246 $[M]^+$ (36), 231 $[M\text{-}CH_3]^+$ (100), 218 $[M\text{-}CO]^+$ (87), 215 $[M\text{-}OCH_3]^+$ (35), 187 (27); HRMS (ESI) calcd for $C_{13}H_{11}O_5$ $[M+H]^+$ 247.0601, found 247.0603.

*3.3.5. 6-Chloro-5-methoxy-2-methyl-1,4-naphthoquinone-2,3-epoxide (**2h**)*

According to the general procedure 3.3.2., compound **2h** was synthesized starting from the naphthoquinone **3h**[24] (41.0 mg, 173 µmol, 1.0 equiv). The residue was purified by column chromatography (*n*-hexane/EtOAc 9:1) to give the epoxide **2h** (29.0 mg, 122 µmol, 71%) as a yellow solid; mp 85 °C; IR (ATR) ν_{max} 2923 (w), 2853 (w), 1699 (s), 1572 (m), 1467 (m), 1404 (m), 1330 (m), 1289 (m), 1240 (s), 1141 (m), 1075 (m), 1029 (m), 965 (s), 851 (m), 750 (m), 717 (s) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (s, 1H, H_{ar}), 7.70 (s, 1H, H_{ar}), 3.97 (s, 3H, OCH_3), 3.86 (s, 1H, CH), 1.72 (s, 3H, 2-CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.8 (C=O), 190.8 (C=O), 155.5 (C-5), 136.6 (C_{ar}), 135.5 (C_{ar}), 132.4 (C_{ar}), 127.0 (C_{ar}), 123.9 (C_{ar}), 62.8 (OCH_3), 62.0 (C-2), 61.7 (C-3), 14.6 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 254 $[M]^+$ (33), 252 $[M]^+$ (91), 239 $[M\text{-}CH_3]^+$ (17), 237 $[M\text{-}CH_3]^+$ (52), 211 (36), 209 (100); HRMS (ESI) calcd for $C_{12}H_9ClNaO_4$ $[M+Na]^+$ 275.0082, found 275.0081.

*3.4. Synthesis of the biotin-labeled analogs **4a/b** and biotin-free compounds **12a/b** and **14a/b** by 'click reaction'*

*3.4.1. Biotinamido-4-pentyne (**7b**)*

To a solution of **5**[37] (100 mg, 320 µmol. 1.0 equiv.) in DMF (8 mL), amino alkyne **6b**[49] (53.2 mg, 640 µmol, 2.0 equiv.) and NEt_3 (160 mg, 219 µL, 1.6 mmol, 5.0 equiv.) was added. After stirring for 14 h the reaction mixture was concentrated in vacuo. Purification by column chromatography (CH_2Cl_2/MeOH 8:1) gave the biotin alkyne **7b** (82.5 mg, 270 µmol, 84%) as a colorless solid; mp 124 °C (CH_2Cl_2/MeOH); $[\alpha]_D^{20}$ +50.9 (MeOH; *c* 0.1); IR (ATR) ν_{max} 3288 (w), 2927 (w), 2853 €, 1696 (s), 1637 (s), 1546 (m), 1460 (m), 1426 (m), 1323 (m), 1265 (m), 1210 (m), 1153 (w), 1074 (m), 1044 (w), 997 (w), 939 (w), 857 (w), 815 (w), 758 (m), 713 (m), 648 (s) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.49 (dd, J = 3.9, 3.1 Hz, 1H, NHCH), 4.30 (dd, J = 7.9, 4.4 Hz, 1H, NHCH), 3.27 (t, J = 6.9 Hz, 2H, $NHCH_2$), 3.24 – 3.17 (m, 1H, SCH), 2.93 (dd, J = 12.8, 5.0 Hz, 1H, SCH_2), 2.71 (d, J = 12.8 Hz, 1H, SCH_2), 2.27 – 2.14 (m, 5H, $C{=}OCH_2$, CH, CH_2), 1.80 – 1.55 (m, 6H, CH_2), 1.49 – 1.39 (m, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.2 (NHC=O), 166.1 ($(NH)_2C$=O), 84.1 (C_q), 70.0 (CH), 63.4 (NHCH), 61.6 (NHCH), 57.0 (SCH), 41.0 (SCH_2), 39.4 ($NHCH_2$), 36.8 (CH_2C=O), 29.8 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 26.9 (CH_2), 26.3 (CH_2) ppm; HRMS (ESI) calcd for $C_{15}H_{23}N_3NaO_2S$ $[M+Na]^+$ 332.1403, found 332.1405.

*3.4.2. 1-Azido-6-bromohexane (**17**)*

To a solution of 1,6-dibromohexane (45.0 g, 185 mmol, 28.2 mL, 1.0 equiv) in DMF (51 mL) and H_2O (12 mL), NaN_3 (13.3 g, 204 mmol, 1.1 equiv) was added. After the reaction mixture was heated at 60 °C for 28 h, the solution was cooled down and then extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give the product **17** (37.5 g, 182 mmol, 98 %) as a colorless liquid; IR (ATR) ν_{max} 2953 (m), 2860 (w), 2089 (s), 1724 (w), 1456 (m), 1348 (w), 1249 (s), 897 (w), 728 (m), 642 (m) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.40 (t, J = 6.8 Hz, 2H, CH_2Br), 3.26 (t, J = 6.8 Hz, 2H, CH_2N_3), 1.86 (m, 2H, CH_2CH_2Br), 1.62 (m, 2H, $CH_2CH_2N_3$), 1.47 (m, 2H, $CH_2(CH_2)_2Br$), 1.39 (m, 2H, $CH_2(CH_2)_2N_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 51.4 (CH_2N_3), 33.8 (CH_2Br), 32.6 (CH_2CH_2Br), 28.8 ($CH_2CH_2N_3$), 27.4 ($CH_2(CH_2)_2Br$), 26.4 ($CH_2(CH_2)_2N_3$) ppm; MS (EI, 70 eV) *m/z* (rel int) 163 $[M\text{-}N_3]^+$ (2), 83 $[M\text{-}BrN_3]^+$ (37), 55 (47), 41 (100).

*3.4.3. 6-[(6'-Bromohexyl)oxy]-2-methyl-1,4-naphthoquinone-2,3-epoxide (**10**)*

Cs_2CO_3 (79.8 mg, 245 µmol, 5.0 equiv) was added with stirring to a mixture of epoxide **2d**[11] (10.0 mg, 49.0 µmol, 1.0 equiv) in acetone (2 mL) at room temperature. After **8** (1.20 g, 750 µL, 4.90 mmol, 100 equiv) was added, the mixture was stirred for 30 min at room temperature. Then the solution was filtered through a short pad of celite and washed with EtOAc. The solvent was removed in vacuo and the residue was purified by column chromatography (*n*-hexane/EtOAc 250:1) to give product **10** (12.8 mg, 34.8 µmol, 71 %) as a colorless oil; IR (ATR) ν_{max} 2927 (m), 2858 (w), 1738 (s), 1693 (s), 1597 (s), 1439 (w), 1366 (m), 1328 (m), 1297 (m), 1230 (s), 1059 (w), 949 (w), 748 (m) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 8.7 Hz, 1H, H_{ar}), 7.35 (d, J = 2.6 Hz, 1H, H_{ar}), 7.20 (dd, J = 8.7, 2.6 Hz, 1H, H_{ar}), 4.16-4.01 (m, 2H, OCH_2), 3.83 (s, 1H, CH), 3.43 (t, J = 6.7 Hz, 2H, $BrCH_2$), 1.97-1.77 (m, 4H, CH_2), 1.72 (s, 3H, CH_3), 1.56-1.44 (m, 4H, CH_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.1 (C=O), 190.8 (C=O), 164.2 (C_{ar}), 134.2 (C_{ar}), 130.1 (C_{ar}), 125.3 (C_{ar}), 122.0 (C_{ar}), 110.4 (C_{ar}), 68.7 (CH_2O), 61.6 (C_qCH_3), 61.4 (CH), 33.9 (CH_2Br), 32.8 (CH_2), 29.0 (CH_2), 28.0 (CH_2), 25.3 (CH_2), 15.0 (CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 367 $[M]^+$ (4), 69 $[C_5H_9]^+$ (17), 57 (24); HRMS (ESI) calcd for $C_{17}H_{19}BrNaO_4$ $[M+Na]^+$ 389.0359, found 389.0352.

*3.4.4. 6-[(6'-Azidohexyl)oxy]-2-methyl-1,4-naphthoquinone-2,3-epoxide (**9**)*

Cs_2CO_3 (2.71 g, 8.20 mmol, 5.0 equiv) was added with stirring to a mixture of epoxide **2d**[11] (335 mg, 1.64 mmol, 1.0 equiv) in acetone (500 mL) at room temperature. After **17** (33.8 g, 164 mmol, 100 equiv) was added, the mixture was stirred for 4 h at room temperature. Then the solution was filtered through a short pad of celite and washed with EtOAc. The solvent was removed in vacuo and the residue was purified by column chromatography (*n*-hexane/EtOAc 500:1) to give product **9** (112 mg, 0.34 mmol, 21 %) as a yellow oil; IR (ATR) ν_{max} 2937 (w), 2861 (w), 2094 (m), 1691 (s), 1596 (s), 1495 (w), 1467 (w), 1438 (w), 1327 (m), 1295 (s), 1236 (m), 1195 (w), 1090 (w), 1060 (w), 1016 (w), 948 (m), 879 (w), 856 (w), 783 (w), 742 (m) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, J = 8.7 Hz, 1H, H_{ar}), 7.35 (d, J = 2.6 Hz, 1H, H_{ar}), 7.20 (dd, J = 8.7, 2.6 Hz, 1H, H_{ar}), 4.11-4.04 (m, 2H, OCH_2), 3.83 (s, 1H, CH), 3.29 (t, J = 6.8 Hz, 2H, N_3CH_2), 1.87-1.79 (m, 2H, CH_2), 1.71 (s, 3H, CH_3), 1.68-1.58 (m, 2H, CH_2), 1.54-1.42 (m, 4H, CH_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.1 (C=O), 190.8 (C=O), 164.2 (C_{ar}), 134.2 (C_{ar}), 130.1 (C_{ar}), 125.3 (C_{ar}), 122.0 (C_{ar}), 110.4 (C_{ar}), 68.7 (CH_2O), 61.6 (C-2), 61.4 (C-3), 51.5 (CH_2N_3), 29.0 (CH_2), 28.9 (CH_2), 26.6 (CH_2), 25.7 (CH_2), 15.0 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 329 $[M]^+$ (4), 258 $[M-C_5H_{11}]^+$ (5), 204 (19), 189 (53), 98 (70); HRMS (ESI) calcd for $C_{17}H_{19}N_3NaO_4$ $[M+Na]^+$ 352.1268, found 352.1264.

3.4.5. General procedure for the 'click reaction'

The reaction was carried out under light exclusion. To a solution of azide **9** (1.0 equiv) in CH_2Cl_2/H_2O (1:1, 170 µL/µmol) $CuSO_4{\cdot}5H_2O$ (1.0 equiv), sodium ascorbate (1.0 equiv) and the respective alkyne in excess were added. After stirring for 2 d at room temperature, the solvent was evaporated.

*3.4.6. Biotinylated epoxide **4a***

According to the general procedure 3.6.3., compound **4a** was synthesized from azide **9** (27.0 mg, 82.0 µmol, 1.0 equiv) and **6a**[37] (27.7 mg, 98.4 µmol, 1.2 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 50:1→10:1) to give product **4a** (8.34 mg, 13.7 µmol, 17 %) as a yellow oil; IR (ATR) ν_{max} 2925 (m), 2854 (m), 2158 (w), 2027 (m), 1978 (m), 1699 (s), 1653 (s), 1593 (m), 1560 (m), 1543 (m), 1523 (m), 1508 (m), 1459 (m), 1289 (s), 1045 (m), 731 (m), 650 (w) cm^{-1}; ^1H NMR (400 MHz, MeOD) δ 7.96 (d, J = 8.7 Hz, 1H, H_{ar}), 7.85 (s, 1H, NCH), 7.36 (d, J = 2.7 Hz, 1H, H_{ar}), 7.29 (dd, J = 8.7, 2.7 Hz, 1H, H_{ar}), 4.48 (dd, J = 7.9, 4.4 Hz, 1H, NHCH), 4.43-4.38 (m, 4H, NCH_2, $NHCH_2$), 4.28 (dd, J = 7.9, 4.4 Hz, 1H, NHCH), 4.11 (t, J = 6.4 Hz, 2H, OCH_2), 3.88 (s, 1H, CH), 3.21-3.15 (m, 1H, SCH), 2.92 (dd, J = 12.7, 4.9 Hz, 1H, SCH_2), 2.72-2.66 (m, 3H, SCH_2, CH_2), 2.23 (t, J = 7.3 Hz, 2H, C=OCH_2), 1.98-1.89 (m, 2H, CH_2), 1.85-1.77 (m, 2H, CH_2), 1.75-1.66 (m, H, CH_2), 1.65 (s, 3H, CH_3), 1.55-1.52 (m, 2H, CH_2), 1.45-1.35 (m, 4H, CH_2) ppm; ^{13}C NMR (100 MHz, MeOD) δ 193.0 (C=O), 192.1 (C=O), 176.0 (NHC=O), 166.1 (C-6), 165.4 ($(NH)_2C$=O), 146.3 (C_qCH_2), 135.5 (C_{ar}), 130.7 (C_{ar}), 126.4 (C_{ar}), 124.3 (NCH), 122.1 (C_{ar}), 111.6 (C_{ar}), 69.7 (OCH_2), 62.7 (C-2), 62.4 (NHCH), 62.3 (CH), 61.6 (NHCH), 57.0 (SCH), 51.3 (NCH_2), 41.1 (SCH_2), 36.5 ($NHCH_2$), 35.6 (C=OCH_2), 31.2 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.4 (CH_2), 27.1 (CH_2), 26.7 (CH_2), 26.4 (CH_2), 14.9 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 551 (5), 523 (5), 367 (5), 97 (17), 71 (19), 69 (23), 57 (33); HRMS (ESI) *m/z* calcd for $C_{30}H_{38}N_6NaO_6S$ $[M+Na]^+$ 633.2466, found 633.2474.

*3.4.7. Biotinylated epoxide **4b***

According to the general procedure 3.6.3., compound **4b** was synthesized from azide **9** (23.3 mg, 70.7 µmol, 1.0 equiv) and **6b** (26.2 mg, 84.8 µmol, 1.2 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 50:1→30:1) to give product **4b** (14.2 mg, 22.2 µmol, 31 %) as an orange oil; IR (ATR) ν_{max} 2925 (s), 2858 (m), 1697 (m), 1596 (w), 1459 (m), 1290 (m), 1127 (s), 1059 (s), 896 (w), 817 (w) cm^{-1}; ^1H NMR (400 MHz, MeOD) δ 1.40-1.48 (m, 4H, CH_2), 1.52-1.61 (m, 4H, CH_2), 1.65 (s, 3H, 2-CH_3), 1.66-1.73 (m, 2H, CH_2), 1.78-1.88 (m, 4H, CH_2), 1.90-1.99 (m, 2H, CH_2), 2.20 (t, J = 7.3 Hz, 2H, C=OCH_2), 2.67-2.74 (m, 3H, SCH_2, CH_2), 2.91 (dd, J = 12.7, 5.0 Hz, 1H, SCH_2), 3.20 (t, J = 6.9 Hz, 3H, $NHCH_2$, SCH), 3.89 (s, 1H, CH), 4.11 (t, J = 6.3 Hz, 2H, OCH_2), 4.29 (dd, J = 7.8, 4.5 Hz, 1H, NHCH), 4.39 (t, J = 7.0 Hz, 2H, NCH_2), 4.48 (dd, J = 7.9, 4.2 Hz, 1H, NHCH), 7.29 (dd, J = 8.7, 2.7 Hz, 1H, H_{ar}), 7.37 (d, J = 2.6 Hz, 1H, H_{ar}), 7.77 (s, 1H, NCH), 7.97 (d, J = 8.7 Hz, 1H, H_{ar}) ppm; ^{13}C NMR (100 MHz, MeOD) δ 14.9 (2-CH_3), 23.6 (CH_2), 26.4 (CH_2), 26.9 (CH_2), 27.1 (CH_2), 29.5 (CH_2), 29.8 (CH_2), 30.2 (CH_2), 30.7 (CH_2), 31.2 (CH_2), 36.8 (C=OCH_2), 39.6 ($NHCH_2$), 41.1 (SCH_2), 51.2 (NCH_2), 57.0 (SCH), 61.6 (NHCH), 62.3 (CH), 62.4 (NHCH), 62.7 (C-2), 69.7 (OCH_2), 111.5 (C_{ar}), 122.1 (C_{ar}), 123.4 (NCH), 126.4 (C_{ar}), 130.7 (C_{ar}), 135.5 (C_{ar}), 147.0 (C_qCH_2), 164.6 ($(NH)_2C$=O), 165.4 (C-6), 176.1 (NHC=O), 191.7 (C=O), 193.0 (C=O) ppm; MS (EI, 70 eV) *m/z* (rel int) 180 (4), 98 (12), 57 (13), 55 (22); HRMS (ESI) *m/z* calcd for $C_{32}H_{42}N_6NaO_6S$ $[M+Na]^+$ 661.2779, found 661.2788.

*3.4.8. Biotin-free derivative **12a***

According to the general procedure 3.6.3., compound **12a** was synthesized from azide **9** (10.0 mg, 30.4 µmol, 1.0 equiv) and 1-pentyne (690 mg, 10.1 mmol, 1.0 mL, 332 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 500:1→50:1) to give product **12a** (2.88 mg, 7.25 µmol, 24 %) as a yellow oil; IR (ATR) ν_{max} 2917 (s), 2852 (m), 1694 (m), 1596 (m), 1465 (s), 1289 (s), 1087 (w), 1058 (w), 737 (m) cm^{-1}; ^1H NMR (400 MHz, MeOD) δ 7.96 (d, J = 8.7 Hz, 1H, H_{ar}), 7.73 (s, 1H, NCH), 7.36

(d, J = 2.6 Hz, 1H, H_{ar}), 7.29 (dd, J = 8.7, 2.6 Hz, 1H, H_{ar}), 4.38 (t, J = 7.0 Hz, 2H, NCH_2), 4.11 (t, J = 6.3 Hz, 2H, OCH_2), 3.88 (s, 1H, CH), 2.65 (t, J = 7.4 Hz, 2H, C_qCH_2), 1. 89-1.89 (m, 2H, CH_2), 1.86-1.77 (m, 2H, CH_2), 1.72-1.65 (m, 2H, CH_2), 1.65 (s, 3H, 2-CH_3), 1.58-1.49 (m, 2H, CH_2), 1.43-1.35 (m, 2H, CH_2), 0.95 (t, J = 7.4 Hz, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, MeOD) δ 193.0 (C=O), 192.1 (C=O), 165.4 (C-6), 149.1 (C_qCH_2), 135.5 (C_{ar}), 130.7 (C_{ar}), 126.4 (C_{ar}), 123.2 (NCH), 122.1 (C_{ar}), 111.5 (C_{ar}), 69.7 (OCH_2), 62.7 (C_qCH_3), 62.4 (CH), 51.1 (NCH_2), 31.2 (CH_2), 28.8 (CH_2), 28.3 (CH_2), 27.1 (CH_2C_q), 26.4 (CH_2), 23.8 (CH_2), 14.9 (2-CH_3), 14.0 (CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 396 $[M-H]^+$ (23), 381 $[M-O]^+$ (14), 369 $[M-CO]^+$ (21), 194 (19), 96 (26), 83 (42), 55 (84); HRMS (ESI) *m/z* calcd for $C_{22}H_{27}N_3NaO_4$ M+Na]$^+$ 420.1894, found 420.1888.

*3.4.9. Biotin-free derivative **12b***

According to the general procedure 3.6.3., compound **12b** was synthesized from azide **9** (27.0 mg, 82.0 µmol, 1.0 equiv) and 1-hexyne (720 mg, 1.0 mL, 8.77 mmol, 107 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 500:1→100:1) to give product **12b** (5.80 mg, 14.1 µmol, 17 %) as a yellow oil; IR (ATR) ν_{max} 2933 (m), 2860 (w), 1692 (s), 1596 (s), 1494 (w), 1439 (m), 1328 (s), 1295 (s), 1229 (s), 1137 (w), 1089 (m), 1059 (m), 948 (m), 856 (w), 743 (m) cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 7.96 (d, J = 8.7 Hz, 1H, H_{ar}), 7.72 (s, 1H, NCH), 7.36 (d, J = 2.6 Hz, 1H, H_{ar}), 7.28 (dd, J = 8.7, 2.6 Hz, 1H, H_{ar}), 4.37 (t, J = 7.0 Hz, 2H, NCH_2), 4.10 (t, J = 6.3 Hz, 2H, OCH_2), 3.88 (s, 1H, CH), 2.67 (t, J = 7.4 Hz, 2H, C_qCH_2), 1.98-1.88 (m, 2H, CH_2), 1.87-1.77 (m, 2H, CH_2), 1.64 (s, 3H, 2-CH_3), 1.68-1.59 (m, 2H, CH_2), 1.58-1.49 (m, 2H, CH_2), 1.44-1.32 (m, 4H, CH_2), 0.93 (t, J = 7.4 Hz, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, MeOD) δ 193.0 (C=O), 192.1 (C=O), 165.4 (C-6), 149.2 (C_qCH_2), 135.5 (C_{ar}), 130.7 (C_{ar}), 126.4 (C_{ar}), 123.1 (NCH), 122.1 (C_{ar}), 111.5 (C_{ar}), 69.7 (OCH_2), 62.7 (C-2), 62.4 (CH), 51.1 (NCH_2), 32.8 (CH_2), 31.2 (CH_2), 29.8 (CH_2), 27.1 (CH_2), 26.4 (CH_2), 26.0 (CH_2C_q), 23.2 (CH_2), 14.9 (2-CH_3), 14.2 (CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 98 (3), 73 (4), 57 (8); HRMS (ESI) *m/z* calcd for $C_{23}H_{29}N_3NaO_4$ [M+Na]$^+$ 434.2050, found 434.2053.

*3.4.10. Biotin-free derivative **14a***

According to the general procedure 3.6.3., compound **14a** was synthesized from azide **9** (23.3 mg, 70.7 µmol, 1.0 equiv) **13a**[38] (8.24 mg, 84.8 µmol, 1.2 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 500:1→50:1) to give product **14a** (10.0 mg, 23.5 µmol, 33 %) as a yellow oil; IR (ATR) ν_{max} 2939 (w), 2862 (w), 1691 (s), 1660 (m), 1596 (s), 1548 (w), 1438 (w), 1329 (m), 1294 (s), 1230 (s), 1058 (w), 948 (w), 856 (w), 742 (w) cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 7.95 (d, J = 8.7 Hz, 1H, H_{ar}), 7.86 (s, 1H, NCH), 7.35 (d, J = 2.6 Hz, 1H, H_{ar}), 7.28 (dd, J = 8.5, 2.5 Hz, 1H, H_{ar}), 4.40 (t, J = 3.5 Hz, 4H, NCH_2, $NHCH_2$), 4.10 (t, J = 6.3 Hz, 2H, OCH_2), 3.88 (s, 1H, CH), 1.96 (s, 3H, 2-CH_3), 1.95-1.89 (m, 2H, CH_2), 1.85-1.76 (m, 2H, CH_2), 1.64 (s, 3H, CH_3), 1.58-1.48 (m, 2H, CH_2), 1.43-1.33 (m, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, MeOD) δ 193.0 (C=O), 192.1 (C=O), 173.2 (NHC=O), 165.4 (C-6), 146.2 (C_qCH_2), 135.5 (C_{ar}), 130.7 (C_{ar}), 126.4 (C_{ar}), 124.2 (NCH), 122.1 (C_{ar}), 111.5 (C_{ar}), 69.6 (OCH_2), 62.7 (C-2), 62.4 (CH), 51.2 (NCH_2), 35.7 ($NHCH_2$), 31.2 (CH_2), 29.8 (CH_2), 27.1 (CH_2), 26.4 (CH_2), 22.4 (CH_3), 14.9 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 398 $[M-CO]^+$ (5), 343 (2), 83 (17), 55 (29); HRMS (ESI) *m/z* calcd for $C_{22}H_{26}N_4NaO_5$ [M+Na]$^+$ 449.1795, found 449.1804.

*3.4.11. Biotin-free derivative **14b***

According to the general procedure 3.6.3., compound **14b** was synthesized from azide **9** (23.3 mg, 70.7 µmol, 1.0 equiv) and **13b**[38] (10.6 mg, 84.8 µmol, 1.2 equiv). The residue was purified by column chromatography on deactivated (NH_3, 7.5% w/w) silica gel (CH_2Cl_2/MeOH 500:1→100:1) to give product **14b** (7.19 mg, 15.8 µmol, 22 %) as a yellow oil; IR (ATR) ν_{max} 2938 (w), 2862 (w), 1691 (s), 1592 (m), 1437 (w), 1371 (m), 1330 (m), 1230 (m), 1174 (m), 1058 (w), 856 (w), 742 (w), 613 (w) cm^{-1}; ^{1}H NMR (400 MHz, MeOD) δ 7.95 (d, J = 8.7 Hz, 1H, H_{ar}), 7.80 (s, 1H, NCH), 7.34 (d, J = 2.7 Hz, 1H, H_{ar}), 7.27 (dd, J = 8.7, 2.7 Hz, 1H, H_{ar}), 4.39 (t, J = 6.9 Hz, 2H, NCH_2), 4.10 (t, J = 6.3 Hz, 2H, OCH_2), 3.88 (s, 1H, CH), 3.73-3.66 (m, 2H, $NHCH_2$), 2.72 (t, J = 7.4 Hz, 2H, C_qCH_2), 2.33 (s, 3H, CH_3), 1.98-1.88 (m, 4H, CH_2), 1.85-1.77 (m, 2H, CH_2), 1.64 (s, 3H, 2-CH_3), 1.58-1.49 (m, 2H, CH_2), 1.42-1.35 (m, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, MeOD) δ 193.0 (C=O), 192.1 (C=O), 175.2 (NHC=O), 165.4 (C-6), 148.1 (C_qCH_2), 135.5 (C_{ar}), 130.7 (C_{ar}), 126.4 (C_{ar}), 123.4 (NCH), 122.1 (C_{ar}), 111.5 (C_{ar}), 69.6 (OCH_2), 62.7 (C_qCH_3), 62.4 (CH), 51.2 (NCH_2), 45.3 ($NHCH_2$), 31.1 (CH_2), 29.8 (CH_2), 29.4 (CH_2), 27.1 (CH_2), 26.4 (CH_3), 26.4 (CH_2), 23.6 (CH_2C_q), 14.9 (2-CH_3) ppm; MS (EI, 70 eV) *m/z* (rel int) 83 (70), 55 (71), 41 (100); HRMS (ESI) *m/z* calcd for $C_{24}H_{31}N_4O_5$ $[M+H]^+$ 455.2289, found 455.2293.

3.5. Cell culture

The human MM cell line INA-6 was a gift from Dr. Martin Gramatzki (Kiel, Germany). Cells were cultured in a humidified incubator at 37°C and 5% CO_2, in RPMI-1640 medium (Sigma, Deisenhofen, Germany) supplemented with 2 ng/mL interleukin 6, 10% FBS (PAA part of GE Healthcare, Pasching, Austria), 100 U/ml penicillin, 100 µg/mL streptomycin (Sigma Aldrich GmbH), 1 mM sodium pyruvate (Sigma Aldrich), and 2 mM L-glutamine (Sigma).

3.6. Viability assessment

Viability was determined by staining with propidium iodide (PI) and annexin V labelled with fluoresceinisothiocyanate (FITC) as described before.[50] Briefly, 8,000 INA-6 cells were seeded and treated with the respective drug concentrations for 3 days INA-6 cells were washed with PBS buffer, incubated in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) containing 2.5 mL annexin V-FITC and 1 mg/mL PI, and analyzed by flow cytometry (FACSCalibur/CELLQuest; Becton Dickinson, Heidelberg, Germany). Thereby, Annexin V was prepared following a known[51] protocol and coupled to FITC (Sigma, Deisenhofen, Germany; F725). Whereas the early apoptotic stage can only be detected by binding of annexin V to translocated phosphatidylserine residues at the external cell membrane, later apoptotic stages, in which cellular membrane integrity is lost, can additionally be visualized by incorporation of the DNA-binding agent PI. Thus, the cell fraction that is negative for both annexin V-FITC and PI is considered viable. On the basis of the respective INA-6 viable cell fractions, the means, standard deviations, dose−response curves, and EC_{50} values for compounds **2a-h**, **4a**, **4b**, **9**, **10**, **12a**, **12b**, **14a** and **14b** were calculated using GraphPad Prism software version 7 (GraphPad Software Inc., La Jolla, USA).

3.7. Drug target identification

3.7.1. Magnetic bead preparation

Pierce streptavidin magnetic beads (catalog no. 88816, Thermo) and biotinylated inhibitor in DMSO were equilibrated to room temperature. For each sample, 50 µL of bead slurry was washed with 500 µL of a 1:1 mixture of DMSO and 50 mM borate buffer at pH 8.5. To saturate the beads with the inhibitor, we used 500 nmol of the biotinylated inhibitor in 400 µL of a 1:1 mixture of DMSO and 50 mM borate buffer at pH 8.5. Streptavidin beads and biotinylated inhibitor were incubated (under rotation) overnight at room temperature. Control beads were prepared without inhibitor accordingly. The supernatant was discarded, and the beads were washed twice with 1 mL of 50% DMSO. After a second wash, the bead slurry was transferred to a new reaction tube and was washed again twice with 500 µL of Pierce IP lysis buffer (catalog no. 87787, Thermo).

3.7.2. Cell lysis

INA-6 cells (5 × 107) were lysed with 2 mL of IP Pierce lysis buffer together with 20 µL of Halt protease inhibitor cocktail (catalog no. 78430, Thermo Fisher Scientific, Massachusetts, USA). Cells were incubated on ice for 10 min with periodic mixing. Lysates were cleared by centrifugation at 16,000g at 4 °C for 5 min, and protein concentration was determined (BCA Protein Assay, Thermo Fisher Scientific).

3.7.3. Drug target enrichment

Inhibitor-loaded beads and control beads were incubated with cleared INA-6 cell lysate (5 mg total protein) for 3 h at 4 °C and overhead rotation. Beads were washed four times with 1 mL of 20 mM HEPES buffer at pH 7.5, 115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4, and 0.5% NP-40. Proteins were eluted with 360 µL of 1× lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and reduced by adding 40 µL of 500 mM DTT and boiling the sample for 10 min at 70 °C. Samples were alkylated with a final concentration of 120 mM iodoacetamide at room temperature in the dark. The eluates were separated from the beads with a magnet. Proteins were precipitated by adding the 4-fold sample volume of acetone. Precipitation was performed overnight at −20 °C. Pellets were washed three times with 1 mL of acetone.

3.7.4. In-solution digestion

Precipitated proteins were dissolved in 0.5% sodium deoxycholate (SDC, Sigma-Aldrich) in 100 mM ammonium hydrogen carbonate. Digests were performed with trypsin (trypsin-to-protein ratio 1:200) overnight at 37 °C. SDC was removed by extraction with ethyl acetate.[52] Peptides were dried in a vacuum concentrator (Concentrator 5301, Eppendorf) to remove remaining ethylaceate. Peptides were desalted using C18 stage tips.[53] Each stage tip was prepared with three disks of C18 Empore SPE disks (3M) in a 200 µL pipet tip. Peptides were eluted with 80% acetonitrile/0.1% formic acid, dried in a vacuum concentrator, and stored at −20 °C. Peptides were dissolved in 2% acetonitrile/0.1% formic acid prior to nanoLC-MS/MS analysis.

3.8. NanoLC-MS/MS analysis

NanoLC-MS/MS analyses were performed on an Orbitrap Fusion (Thermo) equipped with an EASY-Spray ion source and coupled to an EASY-nLC 1000 (Thermo). Peptides were loaded on a trapping column (2 cm × 75 µm ID, PepMap C18, 3 µm particles, 100 Å pore size) and separated on EASY-Spray analytical columns (75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size reverse phase material) with 200 nL/min flow and linear gradients from 3% to 32% acetonitrile/0.1% formic acid.

In-solution digests were analyzed with 50 cm analytical columns and a 180 min gradient (MS scan resolution of 60,000, MS/MS scan resolution 7,500, HCD fragmentation, and top speed method with a maximum 3 s cycle time).

Singly charged precursors were excluded from selection, and a dynamic exclusion list was applied. EASY-IC was used for internal calibration for all runs.

3.9. MS data analysis

For MS raw data file processing, database searches, and quantification, MaxQuant version 1.5.3.30 was used.[54] Searches were performed against the *H. sapiens* reference proteome database (UniProt) and additionally, a database containing common contaminants. The search was performed with tryptic cleavage specificity with three allowed miscleavages.

Protein identification was under the control of the false-discovery rate (<1% FDR on protein and peptide level). In addition to MaxQuant default settings, the search was performed against the following variable modifications: protein N-terminal acetylation, Gln to pyro-Glu formation (N-term. Gln), and oxidation (Met). For protein quantification, LFQ intensities were used.[55] Protein groups with less than two identified razor/unique peptides were dismissed. Experiments for which in-solution and in-gel digests were performed were combined as technical replicates during analysis.

For further data analysis, in-house developed R scripts were used. Missing LFQ intensities in the control samples were imputed with values close to the baseline, i.e., with values from a standard normal distribution with a mean of the 1% quantile of the log10-transformed LFQ intensities (of inhibitor and corresponding control sample) and a standard deviation of 0.05. Significantly enriched proteins were identified by using robust statistics based on intensity binned boxplots (at least 200 proteins per bin), proteins outside the 1.5- or 3-fold interquartile range (IQR) of the distribution were classified as significant (1) or extreme significant (2) outliers, respectively.

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Supplementary data

Supplementary data including NMR spectra (^1H, ^{13}C) and detailed results from affinity capture experiments associated with this article can be found, in the online version, at http://dx.doi.org/10.1017/j.tet.2018.xx.xxx.

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