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Bioconjugation

Azidopropylvinylsulfonamide as a New Bifunctional Click Reagent for Bioorthogonal Conjugations: Application for DNA–Protein Cross-Linking

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Abstract: *N*-(3-Azidopropyl)vinylsulfonamide was developed as a new bifunctional bioconjugation reagent suitable for the cross-linking of biomolecules through copper(I)-catalyzed azide–alkyne cycloaddition and thiol Michael addition reactions under biorthogonal conditions. The reagent is easily clicked to an acetylene-containing DNA or protein and then reacts with cysteine-containing peptides or proteins to form covalent cross-links. Several examples of bioconjugations of ethynyl- or octadiynyl-modified DNA with peptides, p53 protein, or alkyne-modified human carbonic anhydrase with peptides are given.

Introduction

Biorthogonal conjugation reactions are an indispensable tool for the modification of biomolecules (i.e., proteins, nucleic acids, lipids, or glycans).[1] Many of these bioconjugation reactions can even be performed in living cells to monitor biological processes in real time.[2] In particular, bioconjugation of nucleic acids with peptides, proteins, or lipids has found many applications in medicinal chemistry, chemical biology, and nanotechnology.[3] These biomolecules are usually connected to either the 3′- or the 5′-terminus of an oligodeoxynucleotide (ON),[4] but it is highly desirable to attach them to nucleobases to allow control of the position(s) and number of modifications. Recent advances in oligonucleotide synthesis (either chemical phosphoramidite[5] or enzymatic synthesis by using base-modified 2′-deoxynucleoside triphosphates[6] (dNTPs)) has enabled the introduction of many reactive groups into DNA (e.g., aldehyde,[7] alkyne,[8,9] alkene,[10] diene,[11] azide,[12] or tetrazole[13]). These reactive DNA probes were subsequently further modified by reductive amination,[14] [2+3]-dipolar cycloadditions,[8,9] Diels–Alder reactions,[15] or Staudinger ligation.[16] Reactive base-modified DNA has also enabled the crystal structures of several proteins with DNA targets to be solved by covalent trapping of protein cysteine (Cys) mutants by using disulfide cross-linking.[17] Recently, two new DNA–protein conjugation strategies were developed: reductive amination of aldehyde-modified DNA with lysine or arginine[18] and photocross-linking of DNA containing diazirine with various nucleophilic amino acids.[19] The former method requires the use of sodium borohydride as an external reducing agent, which limits its application in cells, whereas the latter is nonspecific. We developed vinylsulfonamide (VS) as a reactive group for the construction of modified DNA probes by enzymatic synthesis with the corresponding VS-modified dNTP (**dC^{VS}TP**).[20] This Michael acceptor modified DNA was successfully utilized for selective conjugation with tumor suppressor protein p53 through the thiol group of Cys by taking advantage of the proximity effect. Although this highly reactive group can be incorporated by polymerase synthesis, it would not be compatible with the phosphoramidite protocol for the chemical synthesis of modified ONs on a solid support. Therefore, we envisaged the possibility of installing the Michael acceptor moiety postsynthetically through a suitable bifunctional reagent. The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC)[21] is the most versatile bioconjugation method and has been extensively used for the modification of oligonucleotides and DNA,[22–25] RNA,[26] proteins,[27,28] or carbohydrates.[29–31] The only known example of an azido-linked Michael acceptor reagent, *N*-3-(azidopropyl)maleimide, was recently described[32] for the cross-linking of two proteins to create a lipase–bovine serum

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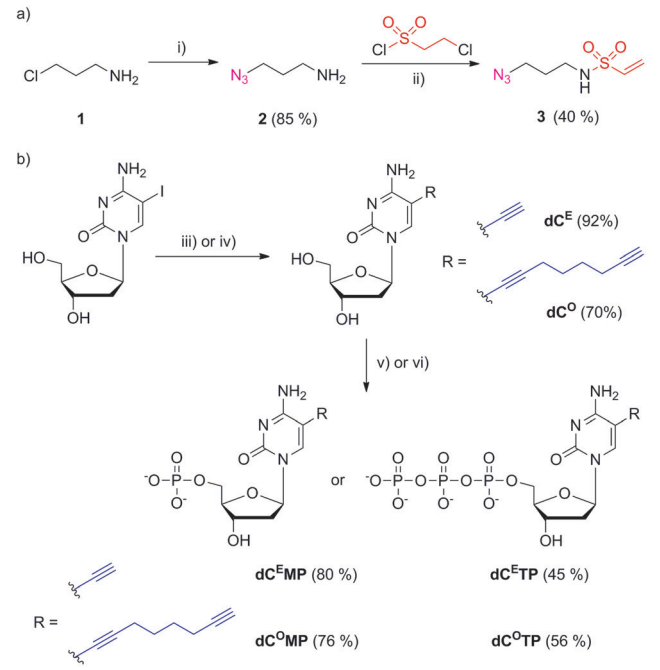
albumin (BSA) heterodimer, but such a reagent would inherently suffer from limited stability of the conjugate, which is susceptible to hydrolysis.[33] Therefore, we designed *N*-(3-azidopropyl)vinylsulfonamide (**3**) as a general bifunctional reagent for the postsynthetic labeling of biomolecules with a reactive VS group.

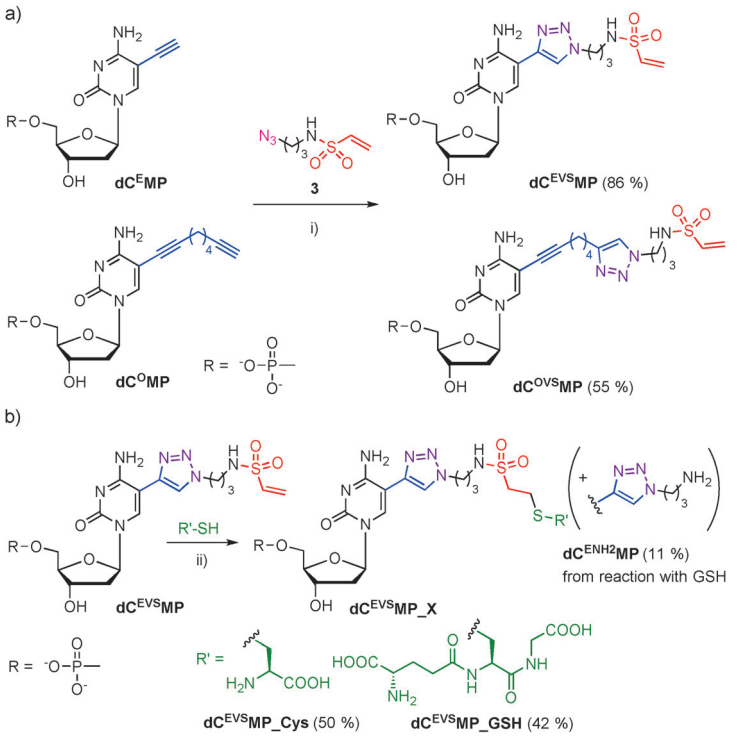
Results and Discussion

Compound **3** was synthesized in two steps starting from 1-chloro-3-aminopropane (**1**; Scheme 1a). Nucleophilic substitution with sodium azide[34] followed by reaction with 2-chloroethanesulfonyl chloride afforded the desired compound **3** in acceptable yield (34% over two steps). Then we intended to test the reactivity of **3** as a new reagent for the CuAAC in a reaction with alkyne-bearing DNA, which could be prepared either on a solid support[8b,9,35] or by enzymatic incorporation of modified dNTPs.[36,37] Therefore, the study required the preparation of 2′-deoxycytidine triphosphates that contained an alkyne moiety at position 5 (**dC^{E}TP** and **dC^{O}TP**) as substrates for the polymerase synthesis of alkyne-modified DNA. They were prepared from 5-iodo-2′-deoxycytidine (Scheme 1b) by Sonogashira coupling with trimethylsilylacetylene (followed by deprotection) or octa-1,7-diyne in a modification of literature protocols.[9,37,38] The reaction temperature for the coupling was lowered to 25 °C (in contrast to previously used[37] 55 °C for **dC^{E}**); this resulted in full conversion in 1 h (compared with previously published times of 3.5 and 24 h[37,38]) and a cleaner reaction mixture. Both alkyne-modified nucleosides were obtained in excellent yields (92% over two steps for **dC^{E}** and 70% for **dC^{O}**). The triphosphorylation of **dC^{E}** and **dC^{O}** with $POCl_3$ in trimethylphosphate followed by treatment with tributylammonium pyrophosphate[36a,37] gave the corresponding compounds **dC^{E}TP** (45%) and **dC^{O}TP** (56%).

To test the bioconjugations, we also needed the corresponding modified 2′-deoxycytidine monophosphates (**dC^{E}MP** and **dC^{O}MP**) as model compounds. They were prepared by phosphorylation of **dC^{E}** and **dC^{O}** in yields of 80 and 76%, respectively (Scheme 1b). The model click reactions were then performed with a twofold excess of bifunctional reagent **3** in the presence of copper(II) sulfate and sodium ascorbate at room temperature (Scheme 2a). In both cases, quantitative conversions to the target Michael acceptor modified dCMPs were observed in 3 h. Final purification by reverse-phase HPLC afforded **dC^{EVS}MP** in good yield (86%). On the other hand, the yield of pure **dC^{OVS}MP** was lower (55%). Therefore, compound **dC^{EVS}MP** was used as a model substrate for Michael additions of Cys and glutathione (GSH) (Scheme 2b). The reactions were performed in triethylammonium acetate (TEAA) buffer (pH 8.3) at 37 °C. In the case of Cys, the conjugation reaction was completed with 1.1 equivalents of Cys in 2 h, whereas a larger excess of GSH (3 equiv) was necessary to reach 90% conversion after overnight incuba-



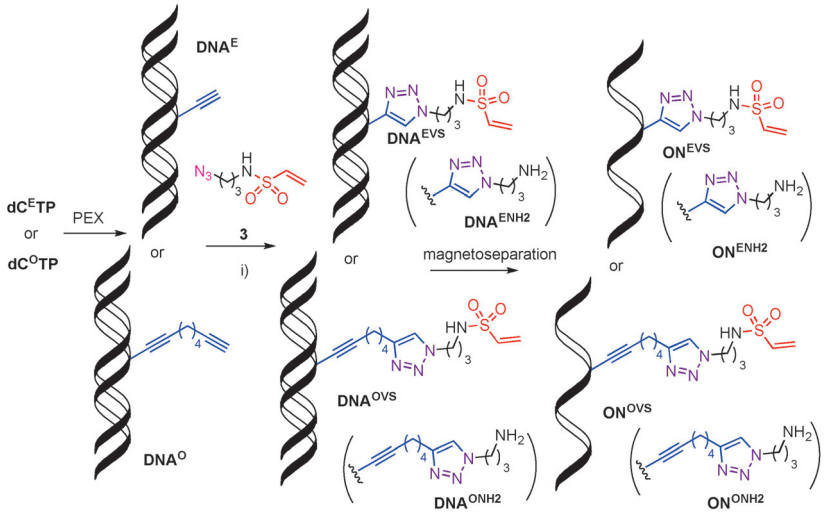
Scheme 1. Syntheses of a) **3** and b) alkyne-modified 2′-deoxynucleosides and nucleotides. Reagents and conditions: i) NaN_3, H_2O, 80 °C, 16 h; ii) 2-chloroethanesulfonyl chloride, diisopropylethylamine (DIPEA), CH_2Cl_2, 0 °C, 1 h; iii) 1) trimethylsilylacetylene, $[PdCl_2(PPh_3)_2]$, CuI, trimethylamine, DMF, 25 °C, 1 h; 2) potassium fluoride, methanol/dioxane, 25 °C, 1.5 h; iv) octa-1,7-diyne, $[PdCl_2(PPh_3)_2]$, CuI, trimethylamine, DMF, 25 °C, 1 h; v) $POCl_3$, $PO(OMe)_3$, 0 °C, 1 h; vi) 1) $POCl_3$, $PO(OMe)_3$, 0 °C, 1 h; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C, 1 h; 3) tetraethylammonium bicarbonate (TEAB; 2 M).



Scheme 2. a) Model click reaction of azide **3** to alkyne-modified dCMPs (**dC^{E}MP**, **dC^{O}MP**); b) Michael addition of L-cysteine and GSH to **dC^{EVS}MP**. Reagents and conditions: i) $CuSO_4$, sodium ascorbate, H_2O/*t*BuOH, 25 °C, 3 h; ii) triethylammonium acetate (TEAA) buffer (0.3 M, pH 8.3), 37 °C.

tion. The products **dC^{EVS}MP_Cys** (50%) and **dC^{EVS}MP_GSH** (42%) were purified by reverse-phase HPLC. In the case of **dC^{EVS}MP_GSH**, product of sulfonamide hydrolysis (**dC^{ENH2}MP**, 11%) was isolated as a byproduct.

Alkyne-modified DNA was prepared by enzymatic incorporation of **dC^{X}TP**s into DNA in a primer extension experiment (PEX; Scheme 3). Both **dC^{E}TP** and **dC^{O}TP** were described to be good substrates for various DNA polymerases (e.g., Vent(*exo*-), Taq, Pwo, KOD XL) in both PEX and PCR.[36a,37] For this study, three DNA polymerases were selected (KOD XL, Vent(*exo*-), and Pwo) and the incorporation was monitored by denaturing polyacrylamide gel electrophoresis (PAGE; Figure S1 in the Supporting Information). All sequences of primer and templates used in this study are listed in Table 1.



Scheme 3. Enzymatic synthesis of alkyne-bearing DNA (**DNA^{X}**) followed by the click reaction of azide **3** and magnetoseparation. Reagents and conditions: i) CuBr/tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA; 1:5), sodium ascorbate, 37 °C, 1 h.

KOD XL polymerase gave clean full-length DNA products that contained either one or two modifications (**DNA^{1X}**, **DNA^{2X}**), which were characterized by MALDI-TOF MS (Table S2 and Figures S10–S13 in the Supporting Information). The modified **DNA^{1E}** and **DNA^{1O}** were then prepared in a larger amount (3 nmol) by PEX with biotinylated template to obtain enough material for the subsequent click reaction, denaturation, and isolation of the corresponding single-stranded ON (ssON) **ON^{1XVS}** by magnetoseparation[39] for MALDI-TOF analysis. The PEX products were purified by using a QIAquick nucleotide removal kit (QIAGEN) to remove unincorporated dNTPs. At first, the click reaction of **DNA^{1X}** with azide **3** was performed under commonly used conditions,[35] that is, in water/DMSO/*t*BuOH, with CuBr as a source of Cu^{I}, TBTA as a Cu^{I}-stabilizing ligand,[40] and sodium ascorbate, which was proven to have significant effect to achieve the best results[25] (Scheme 3). MALDI-TOF analysis confirmed full conversion to **ON^{1XVS}**, but the product was always accompanied by certain amount of **ON^{1XNH2}** (Scheme 3) as a product of partial hydrolysis of sulfonamide (for copies of the MALDI-TOF mass spectra, see Figures S14 and S15 in the Supporting Information). To evaluate whether the product was hydrolyzed during the click reaction or in the mass spectrometer, VS-modified cytidine triphosphate, **dC^{EVS}TP**, was prepared through the click reaction of **dC^{E}TP** with **3** (Scheme S3b in the Supporting Information). After incorporation of **dC^{EVS}TP** into DNA by PEX with a biotinylated template and subsequent magnetoseparation, the resulting **ON^{1EVS}** was analyzed by MALDI-TOF MS (for a copy of the spectrum, see Figure S18 in the Supporting Information). No signal of the hydrolyzed product (**ON^{1ENH2}**) was observed, which indicated that partial decomposition occurred during the click reaction.

Therefore, the conditions of CuAAC were further optimized (all examined conditions are summarized in Table S4 in the Supporting Information). The ratio of DNA to azide **3**, and Cu^{I} to ligand (either commercially available TBTA or next generation, water-soluble bis[(*tert*-butyltriazoyl)methyl][(2-carboxymethyltriazoyl)methyl]amine (BTTAA);[41] for structures, see Scheme S1 in the Supporting Information), were varied for both **DNA^{1E}** and **DNA^{1O}**. In parallel, the influence of reaction time, temperature, and solvent effect were tested (water or phosphate-buffered saline (PBS), pH 7.4 were used instead of a mixture of water/DMSO/*t*BuOH).[12b,25] Unfortunately, the formation of **ON^{1XNH2}** byproduct was observed in all cases (Table S4 in the Supporting Information). Based on all of these observations, all further click reactions were performed with 5 μM **DNA^{1E}**, an excess of **3** (400 equiv), CuBr/TBTA (1:5, 4 equiv), and sodium ascorbate (2 equiv) in water/DMSO/*t*BuOH (9:3:1) at 37 °C for 1 h; under these conditions, the hydrolysis byproducts were formed in an apparently lowest ratio (as determined by MALDI-TOF MS).

Because mass spectrometry is not a quantitative method, we decided to quantify sulfonamide hydrolysis during the click reaction on ethynyl-modified DNA by using HPLC. To achieve

Table 1. List of oligonucleotides used or synthesized for this study.

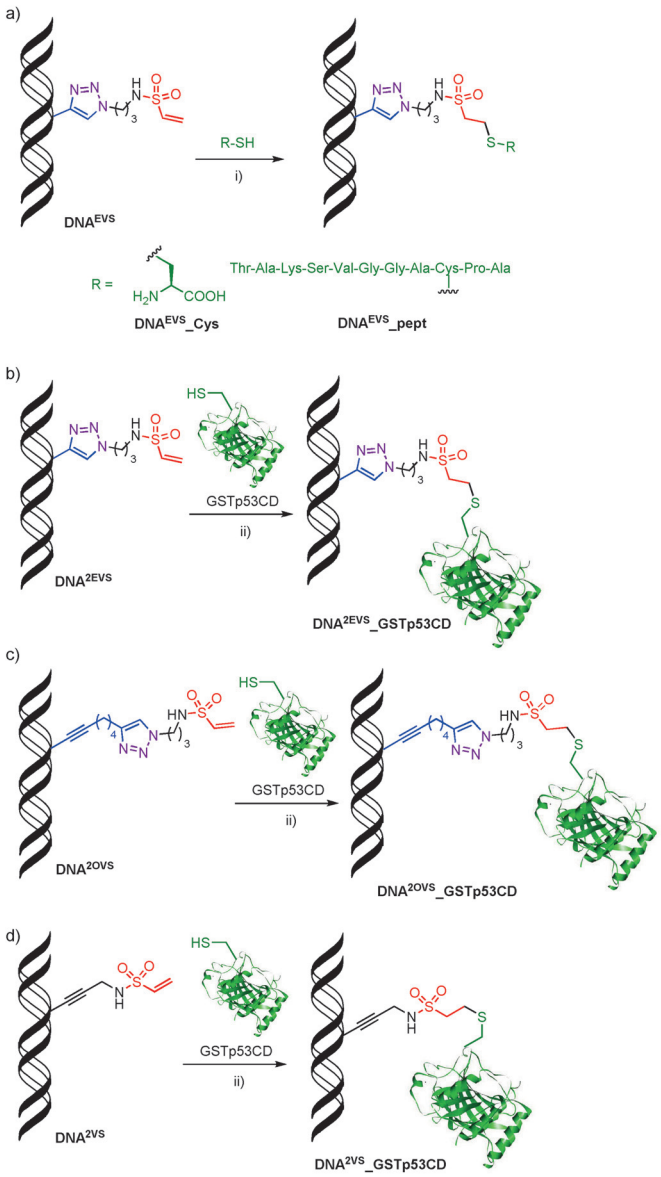
Oligonucleotide	Sequence[a]
prim	5′-TCAAGAGACATGCCT-3′
temp^{1C}	5′-ATAAT*AAACATGTCTAGGCATGTCT*CTTGA-3′
temp^{2C}	5′-ATAAT*AGACATGTCTAGGCATGTCT*CTTGA-3′
ON^{1X}	5′-TCAAG*AGACATGCCTAGA**C**ATGTTT*ATTAT-3′
ON^{2X}	5′-TCAAG*AGACATGCCTAGA**C**ATGT**C**T*ATTAT-3′
DNA^{1X}	3′-AGTTC*TCTGTACGGATCTGTACAA*ATAATA-5′
	5′-TCAAG*AGACATGCCTAGA**C**ATGTTT*ATTAT-3′
DNA^{2X}	3′-AGTTC*TCTGTACGGATCTGTACAG*ATAATA-5′
	5′-TCAAG*AGACATGCCTAGA**C**ATGT**C**T*ATTAT-3′

[a] Italics: p53 recognition sequence. Bold: nucleotides containing modification.

good separation of all three potential click reaction mixture components (**ON^{E}**, **ON^{EVS}**, and **ON^{ENH2}**), it was necessary to use short (ca. 10–12 nucleotides (nt)) starting ssONs (**ON^{E}**). The nicking enzyme amplification reaction (NEAR) was used for their preparation (Scheme S5 in the Supporting Information). This method was recently developed[42] for the enzymatic synthesis of larger amounts (up to 10 nmol) of short base-modified ssONs (10–22 nt). It has been already successfully applied for the preparation of alkyne-modified[25] and fluorescent[43] primers for the polymerase chain reaction (PCR). The template for NEAR was designed to obtain the 10 nt ssON containing one modified cytidine (for sequences of both primer and template, see Table S1 in the Supporting Information). Then NEAR was conducted on a semipreparative scale (750 μL) in the presence of Vent(*exo*-) DNA polymerase and Nt.BstNBI nicking enzyme with **dC^{E}TP**, **dC^{ENH2}TP** (for preparation, see Scheme S6 in the Supporting Information), or **dC^{EVS}TP** to obtain ON standards for the product of both the click reaction, **ONnick^{EVS}**, and hydrolysis, **ONnick^{ENH2}** (for copies of MALDI-TOF mass spectra, see Figures S21–23 in the Supporting Information). The product **ONnick^{E}** was then used as a model for the click reaction with **3** under standard conditions. The products were analyzed by HPLC and compared with the HPLC chromatogram of all three authentic standards (Figures S4 and S5 in the Supporting Information). The amount of hydrolyzed byproduct (**ONnick^{ENH2}**) was determined to be about 12% based on signal integral intensity. The formation of hydrolyzed byproduct could, in principle, be overcome by using a copper-free click reaction, for example, stain-promoted azide–alkyne cycloaddition (SPAAC),[2a,44] for DNA modification with bifunctional reagent **3**. Recently, DNA containing cyclooctyne modification was prepared both by enzymatic synthesis[8c] and on a solid support.[8d]

A double-stranded **DNA^{1EVS}** containing Michael acceptor label was then prepared by the click reaction of **DNA^{1E}** with **3** under the above-described conditions and reacted with Cys or model Cys-containing undecapeptide (Scheme 4a). After magnetoseparation, the desired conjugates **ON^{1EVS}_Cys** and **ON^{1EVS}_pept** were successfully characterized by MALDI-TOF MS (for copies of spectra, see Figure S16 and S17 in the Supporting Information). Analogously to our previous study,[20] we tested **DNA^{2XVS}** prepared by the click reaction of either ethynyl- or octadiynyl-modified DNA with **3** in conjugation with the GST-tagged core domain of tumor suppressor p53 (GSTp53CD; Scheme 4b–d).[45] Protein p53 in a complex with its target DNA contains two free Cys residues in close proximity to the oligonucleotide.[46] Cys 277 can be selectively trapped by Michael addition to VS-bearing DNA when the modification is connected to the cytosine through a propargyl linker (**DNA^{2VS}**).[20] The DNA probe was designed to contain two modifications in the p53 binding sequence[47] (Table 1). GSTp53CD was expressed and purified according to a literature protocol.[48]

Then six different DNA probes were prepared: natural DNA; DNA containing alkyne modification (**DNA^{2E}**, **DNA^{2O}**); DNA containing a reactive handle with different linkers, that is, **DNA^{2VS}** (ref. [20]; Scheme 4d), **DNA^{2EVS}** (Scheme 4b), and **DNA^{2OVS}** (Scheme 4c), which were incubated with GSTp53CD. The ability of p53 protein to recognize modified DNA was monitored by 5% native PAGE (Figure 1a), which confirmed that none of the modifications prevented p53 binding (lanes 3–7). Denaturing SDS-PAGE was then performed to investigate whether covalent DNA–protein cross-links had been formed (Figure 1b). The bands with lower mobility confirmed the formation of covalent conjugates (Figure 1b, lanes 3, 5, and 7). The reactivity of new DNA probes prepared by CuAAC (Figure 1b, lanes 5 and 7) was slightly lower than that of the previously used[20] **DNA^{2VS}** (Figure 1b, lane 3), which was probably caused by longer linkers in the clicked adduct (the **C^{VS}** was designed[20] based on the crystal structure of DNA-p53 complex). Probe



Scheme 4. Conjugation of DNA containing Michael acceptor modification with a) L-cysteine and model undecapeptide; b–d) GST-tagged core domain of p53 (GSTp53CD). Reagents and conditions: i) TEAA buffer (0.3 M, pH 8.3), 37 °C; ii) Tris buffer (pH 7.6), 0 °C, 30 min, and then 25 °C, 2 h. (Protein representations generated from PDB 3Q05, ref. [46a].)

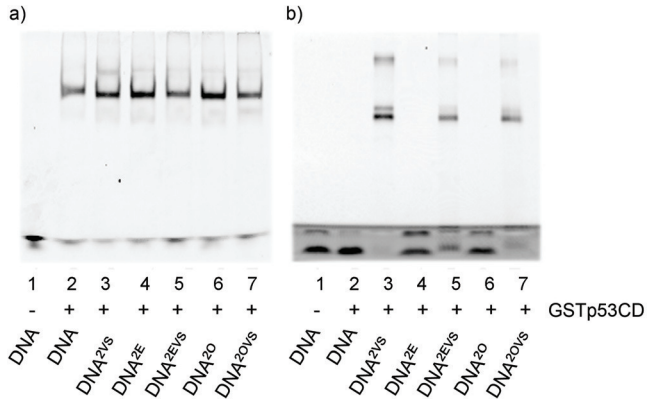


Figure 1. Gel analysis of GSTp53CD binding to various **DNA^{2X}**. a) Native PAGE; b) denaturing sodium dodecyl sulfate (SDS) PAGE. Lane: 1) Natural DNA, 2) natural DNA + GSTp53CD, 3) **DNA^{2VS}** + GSTp53CD, 4) **DNA^{2E}** + GSTp53CD, 5) **DNA^{2EVS}** + GSTp53CD, 6) **DNA^{2O}** + GSTp53CD, 7) **DNA^{2OVS}** + GSTp53CD.

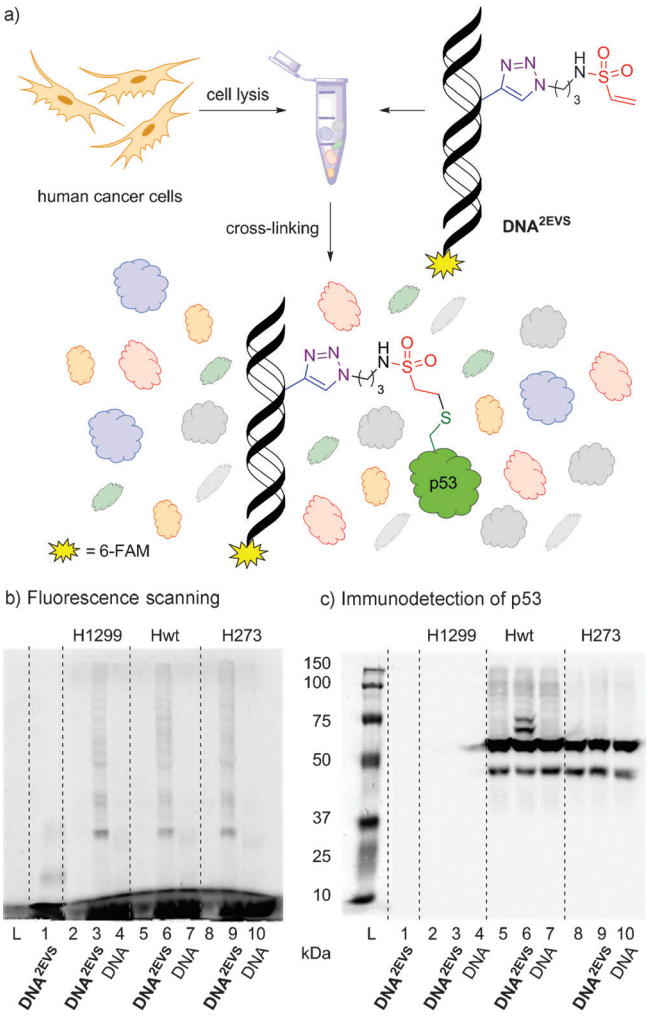


Figure 2. a) Conjugation of p53 from human cancer cells by using reactive **DNA^{2EVS}** probe; b) fluorescence (5′-FAM-labeled DNA) scanning of SDS-PAGE gel; c) western blotting of SDS-PAGE by using anti-p53 antibody. Human nonsmall lung carcinoma cell line H1299 (lanes 2–4): p53 null; Hwt (lanes 5–7): wild-type p53; H273 (lanes 8–10): p53 mutant R273H. Lane L: protein ladder; 1, 3, 6, 9: **DNA^{2EVS}**; 2, 5, 8: no DNA; 4, 7, 10: natural DNA. Conditions: cell lysate (total protein amount of 2.5 μg/μL), DNA (2.5 ng/μL), Tris buffer (pH 7.6), 0 °C, 30 min, and then 25 °C, 2 h.

DNA^{2EVS} was selected as a candidate suitable for trapping p53 from cellular lysates.

Recently, the group of Davis described a method for the analysis of cellular DNA-binding proteins with respect to the presence of C or 5-hydroxymethyl-C when using diazirine-containing reactive DNA probes.[19] These probes were incubated with nuclear extract of HeLa cells and nonspecifically photo-cross-linked to DNA-binding proteins. Herein, we aimed to develop a complementary method, in which only proteins that bound to DNA and contained a free Cys side chain in close proximity to the Michael acceptor modification were trapped. We selected three different human nonsmall lung carcinoma cell lines, p53-deficient H1299, and derived stable Tet-inducible cell lines for the expression of wild-type p53 (p53wt) or p53 mutant R273H, which does not specifically bind to DNA.[49] Total cell lysates were incubated with 5′-FAM-modified **DNA^{2EVS}** (Figure 2a; FAM = fluorescein) and the formation of DNA–protein cross-links was monitored by denaturing PAGE. Fluorescence scanning by Typhoon FLA 9500 (Figure 2b) proved the conjugation of many various cellular proteins from all cell lines to our reactive **DNA^{2EVS}** probe (Figure 2b, lanes 3, 6, and 9). This opens the door to many applications of these probes in proteomics or in the synthesis of irreversible inhibitors of some DNA-processing enzymes. On the other hand, western blotting followed by immunodetection of p53 (Figure 2c) confirmed the **DNA^{2EVS}_p53** conjugate only in cells with induced p53wt (lane 6). Mutant R273H is not trapped by **DNA^{2EVS}** (Figure 2c, lane 9), since the proximity effect is crucial for cross-linking.[20]

The new bifunctional reagent **3** could, in principle, be used for the introduction of the Michael acceptor moiety to any other alkyne-containing biomolecule. To verify its potential in protein modification, human carbonic anhydrase II (hCA)-containing terminal acetylene in a specific position was selected as a model protein.[50] In the past few years, several methods have been developed to introduce unnatural amino acids (UAA) into proteins.[51] One of the most powerful approaches for incorporating UAA site-specifically into proteins expressed in cells is genetic code expansion.[52] A wide range of amino acids can be inserted into proteins by using this methodology, which enables the expansion of chemical biology.[53] Herein, the noncanonical amino acid containing alkyne in its side chain, that is, N^6-[(prop-2-yn-1-yloxy)carbonyl]-L-lysine (**4**), was introduced into position 131 of hCA by following the procedure described by Carell and co-workers.[50] The modified hCA (**hCA***) was expressed in *Escherichia coli* by using the amber codon suppression and orthogonal pyrrolysyl-tRNA/pyrrolysyl-tRNA synthetase system (Figure 3).[54,55] The modified amino acid **4** was prepared according to a previously published procedure from α-Boc-protected lysine (Boc = *tert*-butyloxycarbonyl; Scheme S2 in the Supporting Information).[56] The structure of the modified protein was confirmed by measuring MALDI-TOF mass spectra (*m/z* calcd for $[M+H]^+$: 29236 Da; found: 29238 Da; Figure S27 in the Supporting Information). The CuAAC reaction with **hCA*** was first optimized by using azido-

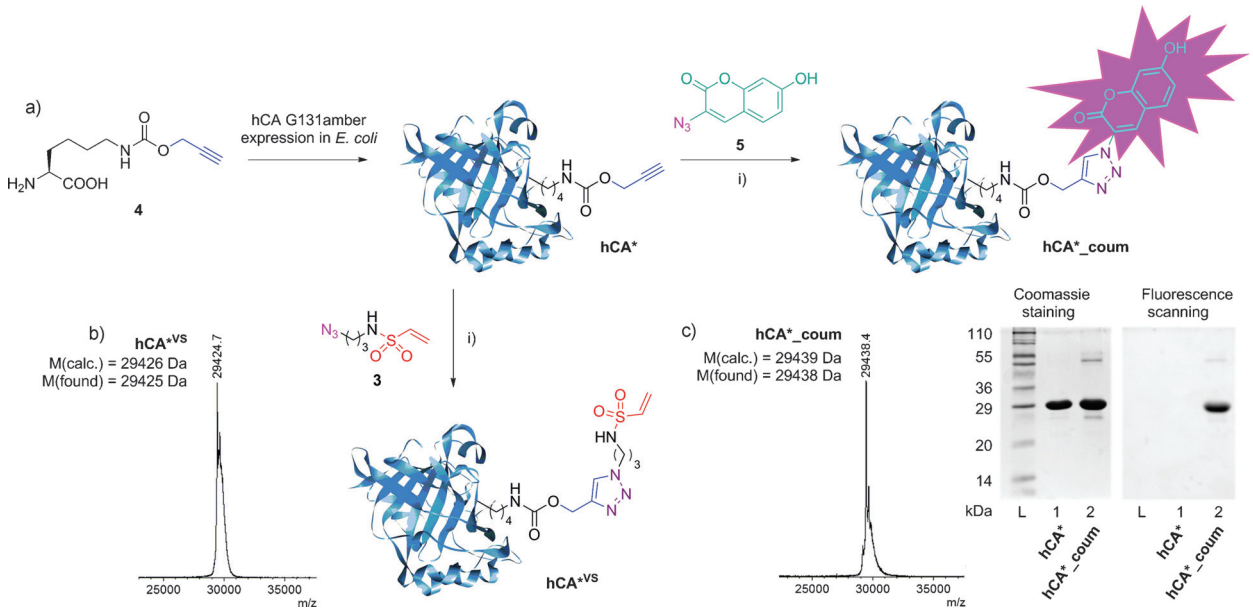


Figure 3. a) Preparation of hCA containing alkyne-modified amino acid **4** at position 131 (**hCA***) followed by the click reaction with coumarin azide **5** or new bifunctional reagent **3**. b) MALDI-TOF mass spectrum of **hCA*^{VS}**. c) MALDI-TOF mass spectrum of **hCA*_coum**; SDS-PAGE gel analysis of reaction of **hCA*** with coumarin **5** visualized by both standard Coomassie staining and fluorescence scanning. Conditions: i) $CuSO_4$/3-[4-({bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propanol (BTTP; 1:2), sodium ascorbate, 30 °C, 1 h. (Protein representations generated from PDB 2VVB.[58])

coumarin[57] **5** as a substrate (Figure 3). The resulting triazolyl–coumarin is fluorescent, and therefore, the formation of **hCA*_coum** can be easily monitored by in-gel fluorescence analysis. The click reaction with azide **5** was performed under the optimized conditions (for details, see Figures S6–S9 in the Supporting Information), that is, with copper(II) sulfate and BTTP[31] ligand (1:2) in the presence of sodium ascorbate at 30 °C for 1 h. The obtained **hCA*_coum** was characterized by measuring MALDI-TOF mass spectra and gel electrophoresis with fluorescent scanning (Figure 3c). Finally, the above-described conditions for the click reaction on protein **hCA*** were applied to the reaction with bifunctional azide **3**. The reaction proceeded smoothly and the structure of target protein **hCA*^{VS}** containing the Michael acceptor handle was proven by mass spectrometry (Figure 3b).

Reactive **hCA*^{VS}** was then incubated with GSH or a model undecapeptide at 37 °C in TEAA buffer (pH 8.3; Figure 4). When GSH was used, complete conversion to Michael adduct **hCA*^{VS}_GSH** was observed and confirmed by mass spectrometry (Figure 4). On the other hand, the longer peptide did not give full conversion to the conjugate **hCA*^{VS}_pept**, which was accompanied by a residual amount of starting **hCA*^{VS}** even after overnight incubation (Figure 4).

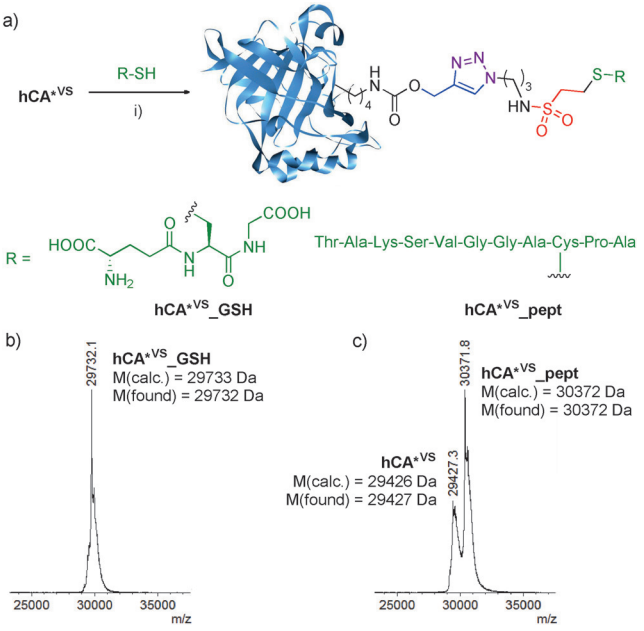


Figure 4. a) Conjugation of hCA containing Michael acceptor modification (**hCA*^{VS}**) with GSH and model undecapeptide. b) MALDI-TOF mass spectrum of **hCA*_GSH**. c) MALDI-TOF mass spectrum of **hCA*_pept**. Conditions: i) TEAA buffer (0.3 M, pH 8.3), 37 °C, overnight.

Conclusion

We developed *N*-(3-azidopropyl)vinylsulfonamide **3** as a new bifunctional bioconjugation reagent. It could be clicked to an alkyne-modified DNA or protein by the CuAAC reaction. Although we showed that partial hydrolysis (ca. 12%) of the sulfonamide occurred in the click modification of DNA, it could still be used for efficient biorthogonal modification of biomolecules to attach VS as a Michael acceptor. Because alkyne (ethynyl or octadiynyl)-linked ONs and/or DNA are easily accessible and even commercially available, this postsynthetic modification is significantly more practical than the direct synthesis of ONs/DNA with reactive nucleotides. Also, the expression of alkyne-modified proteins is now a routine procedure and their conversion into a Michael acceptor handle is simple and

useful. The VS-linked DNA or protein reacts specifically with Cys-containing peptides and/or proteins to form stable covalent cross-links. We believe that this approach will also work for any other combination of two biomolecules when one contains an alkyne and the other one a thiol. Clearly, there is great potential in pull-down experiments for the identification and isolation of DNA-binding proteins containing Cys near to recognition sequences. Studies in this direction are under way in our laboratories.

Experimental Section

General

NMR spectra were recorded on a 600 (600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C), 500 (499.8 or 500.0 MHz for ^{1}H, 202.3 or 202.4 MHz for ^{31}P, 125.7 MHz for ^{13}C), or 400 MHz (400.0 MHz for ^{1}H, 162 MHz for ^{31}P, 100 MHz for ^{13}C) spectrometer from solutions in D_2O, $CDCl_3$, or CD_3OD. Chemical shifts (in ppm, δ scale) were referenced as follows: D_2O (referenced to dioxane as an internal standard: $\delta=3.75$ ppm for ^{1}H NMR spectroscopy and $\delta=69.30$ ppm ^{13}C NMR spectroscopy); CD_3OD (referenced to solvent signal: $\delta=3.31$ ppm for ^{1}H NMR spectroscopy and $\delta=49.00$ ppm for ^{13}C NMR spectroscopy), $CDCl_3$ (referenced to solvent signal: $\delta=7.26$ ppm for ^{1}H NMR spectroscopy and $\delta=77.16$ ppm for ^{13}C NMR spectroscopy). Coupling constants (*J*) are given in Hz. Complete assignment of all NMR spectroscopy signals was achieved by using a combination of H,H-COSY, H,C-HSQC, and H,C-HMBC experiments. Mass spectra and high-resolution mass spectra were measured by using the ESI ionization technique. Chemicals were purchased from Sigma-Aldrich, Stem Chemicals, Berry&Associates, or Acros Organics and used without further purification. The water used in synthetic procedures was of HPLC quality; in biochemical experiments of ultrapure quality (18 MΩ cm). The MALDI-TOF spectra were measured on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with a 1 kHz smartbeam II laser. The measurements were done in reflectron mode by means of the droplet technique, with a mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in a ratio of 9/1/1. The matrix (1 μL) was applied on the target (ground steel) and dried at room temperature. The sample (1 μL) and matrix (1 μL) were mixed and added on the top of the dried matrix preparation spot and dried at room temperature. UV/Vis spectra were measured on a NanoDrop1000 (ThermoScientific) spectrometer at room temperature. Samples were concentrated on a CentriVap Vacuum Concentrator system (Labconco). Synthetic oligonucleotides (primers, templates, and biotinylated templates; for sequences see Table S1 in the Supporting Information) were purchased from Generi Biotech. Natural nucleoside triphosphates (dATP, dGTP, dTTP, dCTP), Vent(*exo*-) DNA polymerase, and nickase Nt.BstNBI were purchased from New England Biolabs; KOD XL DNA polymerase from Merck; streptavidine magnetic particles from Roche; QIAquick nucleotide removal kit from Qiagen; and BioTrace NT nitrocellulose transfer membrane from Pall. Monoclonal mouse anti-p53 antibody DO-1 was kindly provided by Dr. Vojtesek (Masaryk Memorial Cancer Institute, Brno, Czech Republic), antimouse IgG horseradish peroxidase (HRP)-linked antibody was purchased from Sigma-Aldrich. Amersham ECL western blotting detection reagent was purchased from GE Healthcare. Syntheses and characterization data for 3-azido-7-hydroxycoumarin,[57] **4**,[56] BTTAA,[41] BTTP,[31] and **dC^{VS}TP**[20] were reported previously. GSTp53_CD was expressed in *E. coli* cells and purified according to a procedure reported in the literature.[48] 1-Amino-3-azidopropane (**2**),[34] **dC^{E}**,[37,38] **dC^{O}**,[9] **dC^{E}TP**,[37] and **dC^{O}TP**,[36a] were synthesized according to procedures reported in the literature, as described in the Supporting Information.

N-(3-Azidopropyl)vinylsulfonamide (3)

1-Amino-3-azidopropane (**2**, 250 mg, 2.50 mmol) and *N*,*N*-diisopropylethylamine (1 mL, 6.25 mmol) were dissolved in dry dichloromethane (7 mL) on ice under an argon atmosphere. 2-Chloroethanesulfonyl chloride (0.25 mL, 2.50 mmol) was added dropwise and the resulting mixture was stirred at 0 °C for 1 h. The reaction was quenched by the addition of brine (5 mL). The organic phase was washed with brine (10 mL) and water (10 mL) and then dried over $MgSO_4$. The crude product was purified by column chromatography (hexane/ethyl acetate, 1:1) to give **3** as a colorless oil (187 mg, 40%). The product was photolabile and stored in the dark. ^{1}H NMR (400 MHz, CD_3OD): $\delta=1.77$ (p, 2H, $J=6.7$ Hz), 3.02 (t, 2H, $J=6.7$ Hz), 3.41 (t, 2H, $J=6.6$ Hz), 5.97 (d, 1H, $J=10.0$ Hz), 6.14 (d, 1H, $J=16.6$ Hz), 6.63 ppm (dd, 1H, $J_1=16.5$, $J_2=10.0$ Hz); ^{13}C NMR (100 MHz, CD_3OD): $\delta=30.4$, 41.0, 49.6, 126.5, 137.6 ppm; MS (ESI$^+$): *m/z* (%): 213.0 (30) $[M+Na]^+$, 403.1 (100) $[2M+Na]^+$, 593.2 (25) $[3M+Na]^+$; HRMS (ESI$^+$): *m/z* calcd for $C_5H_{10}O_2N_4NaS$ $[M+Na]^+$: 213.04167; found: 213.04143.

General procedure A: Preparation of alkyne-modified 2′-deoxycytidine 5′-*O*-phosphates (dC^{X}MP)

Compound **dC^{X}** was dried at 80 °C for 2 h in vacuo. After cooling, dry $PO(OMe)_3$ and $POCl_3$ (1.2 equiv) were added and the reaction mixture was stirred under an argon atmosphere for 1 h on ice. Phosphorylation was stopped by the addition of TEAB (2 M, 1 mL) and water (2 mL). After purification, several codistillations with water and conversion to sodium salt (Dowex 50WX8 in Na^+ cycle), followed by freeze-drying from water, gave the desired **dC^{X}MP** as a white powder.

5-Ethynyl-2′-deoxycytidine 5′-*O*-phosphate (dC^{E}MP): Compound **dC^{E}MP** was prepared according to general procedure A from **dC^{E}** (100 mg, 0.398 mmol) and $POCl_3$ (45 μL, 0.478 mmol) in $PO(OMe)_3$ (1 mL). After purification by C18 reverse-phase HPLC with water/methanol (5 to 100%) containing 0.1 M TEAB buffer as the eluent, product **dC^{E}MP** was isolated in 80% yield (107 mg). ^{1}H NMR (400 MHz, D_2O): $\delta=2.28$ (m, 1H), 2.41 (m, 1H), 3.76 (s, 1H), 3.98 (dd, 2H, $J_1=5.3$, $J_2=4.2$ Hz), 4.16 (m, 1H), 4.50 (m, 1H), 6.23 (t, 1H, $J=6.6$ Hz), 8.20 ppm (s, 1H); ^{31}P{1H} NMR (162 MHz, D_2O): $\delta=2.11$ ppm; MS (ESI$^-$): *m/z* (%): 331.1 (100) $[M+H]^-$, 353.1 (10) $[M+Na]^-$; HRMS (ESI$^-$): *m/z* calcd for $C_{11}H_{13}O_7N_3P$ $[M+H]^-$: 330.04966; found: 330.04877.

5-(Octa-1,7-diynyl)-2′-deoxycytidine 5′-*O*-phosphate (dC^{O}MP): Compound **dC^{O}MP** was prepared according to general procedure A from **dC^{O}** (150 mg, 0.453 mmol) and $POCl_3$ (55 μL, 0.550 mmol) in $PO(OMe)_3$ (1.5 mL). After purification on a DAE Sephadex column (water/2 M TEAB gradient 0–60%), product **dC^{O}MP** was isolated in 76% yield (170 mg, contained 30 mol% of inorganic PO_4^{3-}). ^{1}H NMR (400 MHz, D_2O): $\delta=1.57$–1.79 (m, 4H), 2.19–2.35 (m, 3H), 2.37 (m, 1H), 2.39–2.43 (m, 3H), 4.00–4.10 (m, 2H), 4.19 (m, 1H), 4.48–4.60 (m, 1H), 6.26 (t, 1H, $J=6.7$ Hz), 8.03 ppm (s, 1H); ^{31}P{1H} NMR (162 MHz, D_2O): $\delta=3.36$ ppm; MS (ESI$^-$): *m/z* (%): 410.1 (100) $[M+H]^-$; HRMS (ESI$^-$): *m/z* calcd for $C_{17}H_{21}O_7N_3P$ $[M+H]^-$: 410.11226; found: 410.11191.

General procedure B: Copper-catalyzed click reactions to dC^{X}MP

Alkyne-modified nucleotide, azido derivative (2 equiv), sodium ascorbate (0.4 equiv), and copper(II) sulfate pentahydrate (0.1 equiv) were dissolved in a mixture of water/*tert*-butanol (1:1, 0.5 mL). The reaction mixture was stirred at room temperature for 3 h (complete consumption of the starting material was proved by TLC with propan-2-ol/water/aqueous ammonia (11:2:7) as the eluent). The products were purified by C18 reverse-phase HPLC with a mixture of water/methanol (5 to 100%) containing 0.1 M TEAB buffer as eluent. Several codistillations with water and conversion to the sodium salt (Dowex 50WX8 in Na^+ cycle), followed by freeze-drying from water, gave the desired click reaction products.

dC^{EVS}MP: According to general procedure B, **dC^{EVS}MP** was prepared from **dC^{E}MP** (19.0 mg, 53 μmol), **3** (20.0 mg, 105 μmol), sodium ascorbate (4.2 mg, 21 μmol) and copper(II) sulfate pentahydrate (1.3 mg, 5 μmol) as white solid (24 mg, 86%). ^{1}H NMR (500.0 MHz, D_2O): $\delta = 2.22$ (quin, $J_{2'',1'''} = J_{2'',3'''} = 6.6$ Hz, 2H; H-2′′′), 2.39 (ddd, $J_{gem} = 14.0$, $J_{2'b,1'} = 7.4$, $J_{2'b,3'} = 6.1$ Hz, 1H; H-2′b), 2.45 (ddd, $J_{gem} = 14.0$, $J_{2'a,1'} = 6.3$, $J_{2'a,3'} = 3.4$ Hz, 1H; H-2′a), 3.02 (t, $J_{3'',2'''} = 6.6$ Hz, 2H; H-3′′′), 3.97 (dd, $J_{H,P} = 5.1$, $J_{5',4'} = 3.8$ Hz, 2H; H-5′), 4.21 (qd, $J_{4',3'} = J_{4',5'} = 3.8$, $J_{H,P} = 1.1$ Hz, 1H; H-4′), 4.58 (m, 1H; H-3′), 4.60 (t, $J_{1'',2''} = 6.6$ Hz, 2H; H-1′′′), 6.09 (dd, $J_{2''''b,1''''} = 10.1$, $J_{gem} = 0.7$ Hz, 1H; H-2′′′′b), 6.19 (dd, $J_{2''''a,1''''} = 16.5$, $J_{gem} = 0.$ Hz, 1H; H-2′′′′a), 6.36 (dd, $J_{1',2'} = 7.4$, 6.1 Hz, 1H; H-1′), 6.66 (dd, $J_{1'''',2''''} = 16.5$, 10.1 Hz, 1H; H-1′′′′), 8.30 (s, 1H; H-6), 8.72 ppm (s, 1H; H-5′′); ^{13}C NMR (125.7 MHz, D_2O): $\delta = 32.04$ (CH_2-2′′′), 42.29 (CH_2-3′′′), 42.38 (CH_2-2′), 50.62 (CH_2-1′′′), 66.34 (d, $J_{C,P} = 4.5$, CH_2-5′), 74.04 (CH-3′), 89.08 (CH-1′), 89.31 (d, $J_{C,P} = 8.4$, CH-4′), 101.99 (C-5), 126.52 (CH-5′′), 131.05 (CH_2-2′′′′), 136.75 (CH-1′′′′), 143.06 (CH-6), 143.93 (C-4′′), 159.23 (C-2), 165.99 ppm (C-4); ^{31}P{^{1}H} NMR (202.4 MHz, D_2O): $\delta = 3.91$ ppm; MS (ESI^-): *m/z* (%): 520.3 (100) $[M+H]^-$, 542.1 (60) $[M+Na]^-$, 564.1 (25) $[M+2Na]^-$; HRMS (ESI^-): *m/z* calcd for $C_{16}H_{23}O_9N_7PS$ $[M+H]^-$: 520.10211; found: 520.10193.

dC^{OVS}MP: According to general procedure B, compound **dC^{OVS}MP** was prepared from **dC^{O}MP** (50.0 mg, 151 μmol), **3** (57.4 mg, 302 μmol), sodium ascorbate (12.0 mg, 60 μmol), and copper(II) sulfate pentahydrate (3.8 mg, 15 μmol) as a white solid (43 mg, 55%). ^{1}H NMR (400.0 MHz, D_2O): $\delta = 1.62$ (m, 2H), 1.78 (m, 2H), 2.12 (m, 2H), 2.29 (m, 1H), 2.48 (m, 3H), 2.76 (t, $J = 7.2$ Hz, 2H), 2.90 (t, $J = 6.7$ Hz, 1H), 3.09 (s, 1H), 3.99 (m, 2H), 4.16 (s, 1H), 4.59–4.42 (m, 3H), 6.09 (m, 1H), 6.25 (m, 1H), 6.60 (dd, $J = 16.5$, 10.1 Hz, 1H), 7.81 (s, 1H), 7.98 ppm (s, 1H); ^{13}C NMR (100 MHz, D_2O): $\delta = 18.5$, 23.9, 26.9, 27.9, 29.2, 39.1, 39.3, 47.2, 64.0, 70.5, 71.0, 85.8, 86.2, 93.3, 97.9, 123.5, 128.4, 133.9, 143.4, 148.3, 156.0, 165.2 ppm; ^{31}P{^{1}H} NMR (161 MHz, D_2O): $\delta = 5.35$ ppm; MS (ESI^-): *m/z* (%): 600.2 (100) $[M+H]^-$; HRMS (ESI^-): *m/z* calcd for $C_{22}H_{31}O_9N_7PS$ $[M+H]^-$: 600.16471; found: 600.16437.

General procedure C: Michael addition of Cys and GSH to dC^{EVS}MP

Modified nucleoside monophosphate analogue (**dC^{XVS}MP**) and L-cysteine (1.1 equiv) or GSH (3 equiv) were dissolved in TEAA buffer (0.3 M, pH 8.3, 0.5 mL) and the mixture was stirred (300 rpm) at 37 °C. The products were purified by C18 reverse-phase HPLC with a mixture of water/methanol (0 to 100%) containing 0.1 M TEAB buffer as the eluent. Several codistillations with water and conversion to the sodium salt (Dowex 50WX8 in Na^+ cycle), followed by freeze-drying from water, gave the desired conjugates.

dC^{EVS}MP_Cys: Compound **dC^{EVS}MP_Cys** was prepared according to general procedure C by stirring **dC^{EVS}MP** (10.0 mg, 19 μmol) with L-cysteine (2.6 mg, 21.2 μmol) for 2 h; full conversion was confirmed by TLC (*i*PrOH/NH_4OH/H_2O, 11:7:2). The product was isolated as a white powder (6 mg, 50%). ^{1}H NMR (600.1 MHz, D_2O): $\delta = 2.24$ (br quin, $J_{2'',1'''} = J_{2'',3'''} = 6.4$ Hz, 2H; H-2′′′), 2.39 (br ddd, $J_{gem} = 13.9$, $J_{2'b,1'} = 6.5$, $J_{2'b,3'} = 6.0$ Hz, 1H; H-2′b), 2.43 (ddd, $J_{gem} = 13.9$, $J_{2'a,1'} = 5.9$, $J_{2'a,3'} = 3.2$ Hz, 1H; H-2′a), 2.96 (br m, 3H; H-2′′′′, 3Cb), 3.08 (br m, 1H; H-3Ca), 3.15 (t, $J_{3'',2'''} = 6.4$ Hz, 2H; H-3′′′), 3.48 (br m, 2H; H-1′′′′), 3.91 (br m, 1H; H-2C), 3.98 (br t, $J_{H,P} = J_{5',4'} = 4.4$ Hz, 2H; H-5′), 4.21 (br td, $J_{4',5'} = 4.4$, $J_{4',3'} = 3.2$ Hz, 1H; H-4′), 4.58 (dt, $J_{3',2'} = 6.0$, 3.2, $J_{3',4'} = 3.2$ Hz, 1H; H-3′), 4.61 (t, $J_{1'',2'''} = 6.4$ Hz, 2H; H-1′′′), 6.35 (dd, $J_{1',2'} = 6.5$, 5.9 Hz, 1H; H-1′), 8.29 (s, 1H; H-6), 8.71 ppm (s, 1H; H-5′′); ^{13}C NMR (125.7 MHz, D_2O): $\delta = 27.52$ (CH_2-2′′′′), 32.32 (CH_2-2′′′), 35.35 (CH_2-3C), 42.34 (CH_2-3′′′), 42.38 (CH_2-2′), 50.55 (CH_2-1′′′), 53.66 (CH_2-1′′′′), 56.35 (CH-2C), 66.40 (d, $J_{C,P} = 4.3$ Hz; CH_2-5′), 73.99 (CH-3′), 89.08 (CH-1′), 89.24 (d, $J_{C,P} = 8.5$ Hz; CH-4′), 101.98 (C-5), 126.53 (CH-5′′), 143.05 (CH-6), 143.92 (C-4′′), 159.20 (C-2), 165.99 ppm (C-4), (C-1C not detected); ^{31}P{^{1}H} NMR (202.4 MHz, D_2O): $\delta = 3.89$ ppm; MS (ESI^-): *m/z* (%): 641.1 (100) $[M+H]^-$, 520.1 (35) $[M+H-Cys]^-$, 663.1 (30) $[M+Na]^-$, 685.1 (10) $[M+2Na]^-$; HRMS (ESI^-): *m/z* calcd for $C_{19}H_{30}O_{11}N_8PS_2$ $[M+H]^-$: 641.12185; found: 641.12146.

dC^{EVS}MP_GSH: Compound **dC^{EVS}MP_GSH** was prepared according to general procedure C by stirring of **dC^{EVS}MP** (15.0 mg, 29 μmol) with GSH (26.6 mg, 86.7 μmol) overnight; conversion of around 90% was confirmed by TLC (*i*PrOH/NH_4OH/H_2O, 11:7:2). Product **dC^{EVS}MP_GSH** was isolated as a white powder (10 mg, 42%) and compound **dC^{ENH2}MP** was obtained as a byproduct (3 mg, 11%). Spectral data for **dC^{EVS}MP_GSH**: ^{1}H NMR (500.0 MHz, D_2O): $\delta = 2.07$ (m, 2H; H-3E), 2.24 (br quin, $J_{2'',1'''} = J_{2'',3'''} = 6.5$ Hz, 2H; H-2′′′), 2.32 (br ddd, $J_{gem} = 14.0$, $J_{2'b,1'} = 7.4$, $J_{2'b,3'} = 6.0$ Hz, 1H; H-2′b), 2.44 (ddd, $J_{gem} = 14.0$, $J_{2'a,1'} = 6.2$, $J_{2'a,3'} = 3.4$ Hz, 1H; H-2′a), 2.44 (m, 2H; H-4E), 2.82 (dd, $J_{gem} = 14.1$, $J_{3b,2} = 8.9$ Hz, 1H; H-3bC), 2.84 (m, 2H; H-2′′′′), 3.03 (m, 1H; H-3Ca), 3.07 (t, $J_{3'',2'''} = 6.5$ Hz, 2H; H-3′′′), 3.37 (m, 2H; H-1′′′′), 3.68 (m, 3H; H-2E, 2G), 3.90 (dd, $J_{H,P} = 5.2$, $J_{5',4'} = 3.9$ Hz, 2H; H-5′), 4.13 (tdd, $J_{4',5'} = 3.9$, $J_{4',3'} = 3.2$, $J_{H,P} = 1.2$ Hz, 1H; H-4′), 4.47–4.55 (m, 4H; H-3′,1′′′,2C), 6.27 (dd, $J_{1',2'} = 7.4$, 6.2 Hz, 1H; H-1′), 8.21 (s, 1H; H-6), 8.62 ppm (s, 1H; H-5′′); ^{13}C NMR (125.7 MHz, D_2O): $\delta =$ 27.5 (CH_2-2′′′′), 29.0 (CH_2-3E), 32.3 (CH_2-2′′′), 34.1 (CH_2-4E), 35.9 (CH_2-3C), 42.4, 42.4 (CH_2-2′,3′′′), 46.1 (CH_2-2G), 50.6 (CH_2-1′′′), 53.8 (CH_2-1′′′′), 55.7 (CH-2C), 56.9 (CH-2E), 66.5 (d, $J_{C,P} = 4.7$ Hz; CH_2-5′), 74.0 (CH-3′), 89.1 (CH-1′), 89.2 (d, $J_{C,P} = 8.2$ Hz; CH-4′), 102.0 (C-5), 126.5 (CH-5′′), 143.1 (CH-6), 143.9 (C-4′′), 159.3 (C-2), 166.1 (C-4), 174.5 (C-1C), 176.9 (C-1E), 177.7 (C-5E), 179.0 ppm (C-1G); ^{31}P{^{1}H} NMR (202.4 MHz, D_2O): $\delta = 3.68$ ppm; MS (ESI^-): *m/z* (%): 520.3 (100) $[M-GSH+H]^-$, 554.1 (98) $[M-GSH+SH+H]^-$, 871.1 (80) $[M+2Na]^-$, 827.2 (70) $[M+H]^-$, 849.2 (50) $[M+Na]^-$, 542.1 (40) $[M-GSH+Na]^-$; HRMS (ESI^-): *m/z* calcd for $C_{26}H_{40}O_{15}N_{10}PS_2$ $[M+H]^-$: 827.18591; found: 827.18469.

Spectral data for **dC^{ENH2}MP**: ^{1}H NMR (600.1 MHz, D_2O): $\delta = 2.35$–2.49 (m, 4H; H-2′,2′′), 2.92 and 2.97 (2×m, 2×1H; H-3′′′), 3.99 (ddd, $J_{gem} = 11.8$, $J_{H,P} = 5.5$, $J_{5'b,4'} = 3.3$ Hz, 1H; H-5′b), 4.03 (ddd, $J_{gem} = 11.8$, $J_{H,P} = 3.6$, $J_{5'a,4'} = 2.6$ Hz, 1H; H-5′a), 4.23 (m, 1H; H-4′), 4.60–4.64 (m, 3H; H-3′,1′′′), 6.39 (dd, $J_{1',2'} = 6.9$, 6.4 Hz, 1H; H-1′), 8.45 (s, 1H; H-6), 8.93 ppm (s, 1H; H-5′′); ^{13}C NMR (125.7 MHz, D_2O): $\delta = 29.7$ (CH_2-2′′), 38.8 (CH_2-3′′′), 42.9 (CH_2-2′), 49.9 (CH_2-1′′′), 66.2 (d, $J_{C,P} = 4.2$, CH_2-5′), 73.8 (CH-3′), 89.0 (CH-1′), 89.5 (d, $J_{C,P} = 8.6$ Hz, CH-4′), 101.9 (C-5), 126.0 (CH-5′′), 143.0 (CH-6), 144.6 (C-4′′), 159.1 (C-2), 165.8 ppm (C-4); ^{31}P{^{1}H} NMR (202.4 MHz, D_2O): 3.85 ppm; MS (ESI^-): *m/z* (%): 430.1 (100) $[M+H]^-$.

Incorporation of dC^XTP into DNA by PEX

The reaction mixture (20 μL) contained primer (0.5 μM), template (0.75 μM), DNA polymerase (0.05 U KOD XL, 0.16 U Vent(*exo*-), or 0.2 U Pwo), dNTPs (either all natural or 3 natural and 1 modified; 20 μM) in enzyme reaction buffer supplied by the manufacturer. Primer was labeled on the 5′-end by 6-carboxyfluorescein (6-FAM). The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler. Primer extension was stopped by the addition of stop solution (2×, 80% (v/v) formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol) and heated for 5 min at 95 °C. Samples were separated by 12.5% PAGE (acrylamide/bisacrylamide 19:1, 25% urea) under denaturing conditions (TBE buffer 1×, 42 mA, 1 h). Visualization was performed by fluorescence imaging by using a Typhoon FLA 9500 instrument from GE Healthcare (Figure S1 in the Supporting Information).

MALDI-TOF analysis of alkyne-modified oligonucleotides (ON^{1E}, ON^{1O}, ON^{2E}, ON^{2O})

The PEX solution (50 μL) contained KOD XL DNA polymerase (0.5 U), primer (4 μM), 5′-biotinylated template (4 μM), and dNTPs (either natural or modified, 264 μM) in KOD XL reaction buffer supplied by the manufacturer. The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler. The reaction was stopped by cooling to 4 °C. Streptavidine magnetic particles (Roche, 60 μL) were washed with binding buffer (3×200 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The PEX solution and binding buffer (50 μL) were added. The mixture was incubated for 30 min at 15 °C and 1200 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3× 500 μL, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (4×500 μL). Then water (50 μL) was added and the sample was denatured for 2 min at 50 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The product was evaporated to dryness, dissolved in a mixture water/acetonitrile (1:1, 5 μL), and analyzed by MALDI-TOF MS (the results are summarized in Table S2 in the Supporting Information; for copies of mass spectra, see Figures S10–13 in the Supporting Information).

Click reaction of azide 3 on DNA^{1E} and DNA^{1O}: Optimized conditions

The DNA^{1X} was prepared by means of a PEX (300 μL) containing KOD XL DNA polymerase (1.25 U), primer (3.3 μM), 5′-biotinylated template $temp^{1C}$ (3.3 μM), and dNTPs (either natural or modified, 264 μM) in KOD XL reaction buffer supplied by the manufacturer. The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler. The products were purified on QIAquick nucleotide removal kit (QIAGEN) and eluted with water. The concentration of DNA product was determined from the absorbance at $\lambda = 260$ nm and the extinction coefficient obtained from an online calculator by IDT Biophysics.[59] The solution of Cu^I catalyst was freshly prepared immediately prior to the reaction by mixing CuBr (0.5 μL, 10 mM in DMSO/*t*BuOH 3:1), TBTA ligand (2.5 μL, 10 mM in DMSO/*t*BuOH 3:1), and DMSO/*t*BuOH (3:1, 47 μL).

The click reaction mixture (40 μL) containing DNA^{1X} (5 μM), azide **3** (2 mM), CuBr/TBTA (1:5; 20 μM Cu^I), and sodium ascorbate (10 μM) in water/DMSO/*t*BuOH 9:3:1 was incubated for 1 h at 37 °C in a thermal cycler.

To use the obtained DNA^{1XVS} in further bioconjugations (with Cys, peptide, p53, or cellular proteins), the product of the click reaction was purified with a QIAquick nucleotide removal kit (QIAGEN) and eluted with water. The concentration of DNA^{1XVS} product was determined from the absorbance at $\lambda = 260$ nm and the extinction coefficient was obtained from an online calculator by IDT Biophysics.[59]

To obtain single-stranded ON^{1XVS} for MALDI-TOF mass analysis, water (60 μL) was added to the click reaction mixture and magnetoseparation was performed. Streptavidine magnetic particles (Roche, 50 μL) were washed with binding buffer (3×200 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The diluted click reaction mixture (100 μL) and binding buffer (100 μL) were added. The mixture was incubated for 30 min at 15 °C and 1200 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3×500 μL, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (4×500 μL). Then water (30 μL) was added and the sample was denatured for 2 min at 50 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The product was evaporated to dryness, dissolved in a mixture water/acetonitrile (1:1, 5 μL), and analyzed by MALDI-TOF MS (for copies of mass spectra see Figures S14 and S15 in the Supporting Information).

Addition of Cys and Cys-containing peptide to DNA^{1EVS} prepared by a click reaction (DNA^{1EVS}_Cys, DNA^{1EVS}_pept)

The reaction mixture (20 μL) contained DNA^{1EVS} (0.4 nmol) and L-cysteine or an undecapeptide (1 μmol) in TEAA buffer (0.3 M, pH 8.3). The solution was incubated overnight at 37 °C in a thermal cycler. Water (40 μL) was added. Streptavidine magnetic particles (Roche, 100 μL) were washed with binding buffer (3×200 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The conjugation reaction mixture (60 μL) and binding buffer (60 μL) were added. The mixture was incubated for 30 min at 15 °C and 1200 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3×500 μL, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (4×500 μL). Then water (30 μL) was added and the sample was denatured for 2 min at 50 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The product was evaporated to dryness, dissolved in a mixture water/acetonitrile (1:1, 5 μL), and analyzed by MALDI-TOF MS (for copies of mass spectra see Figures S16 and S17 in the Supporting Information).

Conjugation of reactive DNA probes (DNA^{2VS}, DNA^{2EVS}, DNA^{2OVS}) with protein p53

Natural DNA, DNA^{2VS}, DNA^{2E}, and DNA^{2O} were prepared by means of a PEX (300 μL) containing KOD XL DNA polymerase (1.25 U), 5′-FAM-labeled primer (3.3 μM), template $temp^{2C}$ (3.3 μM), and dNTPs (either natural or modified, 264 μM) in KOD XL reaction buffer supplied by the manufacturer. The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler. The products were purified with a QIAquick nucleotide removal kit (QIAGEN) and eluted with water. The concentration of DNA product was determined from the absorbance at $\lambda = 260$ nm and the extinction coefficient was obtained from an online calculator by IDT Biophysics.[59]

The DNA^{2EVS} and DNA^{2OVS} were prepared by the click reaction of DNA^{2E} or DNA^{2O} with azide **3** under the optimized conditions described above. The products were purified with a QIAquick nucleotide removal kit (QIAGEN) and eluted with water. The concentration of DNA product was determined from the absorbance at $\lambda =$ 260 nm and the extinction coefficient was obtained from an online calculator by IDT Biophysics.[59]

The reaction mixtures for GSTp53CD protein binding (20 μL) were prepared from purified PEX or click reaction products (10 μL, 6 ng/μL), KCl (500 mM, 2 μL), DTT (2 mM, 2 μL), VP buffer (50 mM Tris, 0.1% Triton-X100, pH 7.6, 2 μL), and GSTp53CD stock solution (700 ng/μL in 25 mM Hepes pH 7.6, 200 mM KCl, 10% glycerol, 1 mM DTT, 1 mM benzamidine; 1 μL). The control sample was prepared analogously without GSTp53CD. All samples were incubated for 30 min on ice, glycerol was added (80%, 2 μL), and part of the reaction mixture (3.5 μL) was separated by use of a 5% native PAGE (acrylamide/bisacrylamide 37.5:1; 0.5×TBE, 4 °C, 80 V/1 h). The rest of the reaction mixture in vials was incubated for 2 h at 25 °C. Loading buffer (5×, 0.3 M Tris·HCl, 5% SDS, 50% glycerol, 2.5% β-mercaptoethanol, 0.05% bromophenol blue) was added and the mixture was heated at 65 °C for 10 min. The samples (10 μL) were separated by 10% SDS-PAGE (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at room temperature (100 V/30 min then 150 V/1 h). Visualization was performed by fluorescence imaging with a Typhoon FLA 9500 instrument from GE Healthcare (Figure 1).

Cell culture and lysis

Human p53 null nonsmall lung carcinoma cell line H1299 (NCI-H1299, ATCC), and derived stable Tet-inducible cell lines for expression of wtp53 or mutp53 (R273H; described in ref. [49]) were grown in Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 5% fetal bovine serum (FBS; FCS; PAA Laboratories GmbH) and penicillin/streptomycin (Gibco) on a 10 cm plate. All cultures were incubated at 37 °C in 5% CO_2. At about 70% confluency, tetracyclin was added to a final concentration of 1 μg mL^{-1} for 24 h to induce p53 prior to cell harvest. Total cell lysates were prepared by cell pellet lysis in 1xPLB, according to the manufacturer's protocol (Promega). The concentration of protein in the lysates was determined by a standard Bradford assay.

Conjugation of DNA^{2EVS} with cellular proteins

The samples (20 μL) containing natural DNA or **DNA^{2EVS}** (5 μL, 6 ng/μL), KCl (500 mM, 2 μL), DTT (2 mM, 2 μL), Tris buffer (50 mM, pH 7.6, 2 μL), and total cell lysates (50 μg of total proteins in 1xPLB buffer). Control samples were prepared analogously without DNA or cellular extracts. All samples were incubated for 30 min on ice, and then for 2 h at 25 °C. Loading buffer (5×, 0.3 M Tris·HCl, 5% SDS, 50% glycerol, 2.5% β-mercaptoethanol, 0.05% bromophenol blue) was added and the mixture was heated at 95 °C for 5 min. The samples (25 μL) were separated by 10% SDS-PAGE (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at room temperature (100 V/30 min then 150 V/1 h). Visualization was performed by fluorescence imaging with a Typhoon FLA 9500 instrument from GE Healthcare (Figure 2b).

The gel was blotted (0.025 M Tris, 0.192 M glycine, 20% methanol; 150 V/1.5 h at 4 °C) on BioTrace NT nitrocellulose transfer membrane (Pall). The membrane was washed with PBS (137 mM NaCl, 2.7 mM KCl,10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), blocked with 5% nonfat milk (in PBS-T, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.5% Tween-20, pH 7.4) for 45 min, and then washed with PBS-T (15 mL, 5 min). The membrane was incubated with monoclonal mouse anti-p53 antibody DO-1 (supernatant provided by Dr. Vojtesek) at a 1:10 dilution in 5% nonfat milk (in PBS-T) at 4 °C overnight. The unbound antibodies were removed by washing with PBS-T (15 mL, 3×5 min). The membrane was incubated with antimouse IgG HRP-linked antibody (Sigma Aldrich) at a 1:10000 dilution in 5% nonfat milk (in PBS-T) at 25 °C for 1 h. The remaining unbound antibody was washed away by TBS-T (20 mL, 4×5 min) and TBS (20 mL, 5 min). The membrane was incubated with Amersham ECL western blotting detection reagent (GE Healthcare) for 1 min. Chemiluminescence was measured (Figure 2c) on an ImageQuant LAS 4000 Mini luminescence analyzer (GE Healthcare).

Expression and purification of hCA II containing alkyne at position 131

The plasmids pACA_HCA_G131amber (hCA under control of the T7 promoter; ampicillin resistance) and pACyc_pylSwt_pylTTT (wtPylRS and three copies of pylT under control of the glutamine promoter from *E. coli*; chloramphenicol resistance) were a kind donation from Prof. Thomas Carell (Ludwig Maximilians University, Munich, Germany).[50] Both plasmids were amplified in *E. coli* DH5α and isolated by using a Zyppy Plasmid Miniprep kit (Zymo Research). The plasmids pACA_HCA_G131amber and pACyc_pylSwt_pylTTT were transformed together into *E. coli* strain BL21(DE3). Cells were grown in LB medium (1 L, 37 °C, 220 rpm) containing modified amino acid **4** (2 mM), carbenicillin (100 mg L^{-1}) and chloramphenicol (25 mg L^{-1}) until OD_{600} = 0.9 was reached. The expression of the HCA G131amber gene was induced by the addition of $ZnSO_4$ (1 mM) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1 mM). After further incubation (10 h, 37 °C, 220 rpm), cells were harvested (3.900 rpm, 10 min, 4 °C) and stored at −20 °C until further use. Cell pellets were resuspended in washing buffer (80 mL; 25 mM Tris, 50 mM Na_2SO_4, 50 mM $NaClO_4$, pH 8.0). After ultrasonic lysis (4×30 s), cell debris was removed by centrifugation (14000 rpm, 45 min, 4 °C). The supernatant was loaded on *p*-aminomethylbenzenesulfonamide–agarose resin (3 mL; Sigma-Aldrich A0796) equilibrated with washing buffer. After protein binding, the column was washed with 10 column volumes of washing buffer. HCA protein was eluted by low-pH buffer (100 mM NaOAc, 200 mM $NaClO_4$, pH 5.6). The fractions were analyzed by 15% SDS-PAGE. The hCA-containing fractions were combined, buffer was exchanged to water by means of a VivaSpin 4 centrifugal concentrator (Vivaproducts), and the sample was freeze-dried. The yield of pure modified hCA protein was 17 mg and its structure was confirmed by measuring of MALDI-TOF mass spectrum (*M* (calcd) = 29236 Da, *M* (found) = 29238 Da $[M+H]^+$; Figure S27 in the Supporting Information).

Copper-catalyzed click reaction on alkyne-modified hCA*: Optimized conditions

The solution of Cu^{II} with BTTP ligand was freshly prepared immediately prior to the reaction by mixing $CuSO_4$ (3.8 μL, 10 mM in water) and BTTP ligand (0.75 μL, 100 mM in DMSO). The click reaction mixture (15 μL) containing **hCA*** (50 μM), azide (1.25 mM), $CuSO_4$/BTTP (1:2; 200 μM Cu^{II}), and sodium ascorbate (500 μM) in water was incubated for 1 h at 30 °C in a thermal cycler.

For gel analysis of **hCA*_coum** formation, part of the reaction mixture (3 μL) was diluted with water (10 μL) and then loading buffer (5×, 0.3 M Tris·HCl, 5% SDS, 50% glycerol, 2.5% β-mercaptoethanol, 0.05% bromophenol blue) was added. The mixture was heated at 95 °C for 5 min. The samples (16.5 μL) were separated by 15% SDS-PAGE (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at room temperature (100 V/1 h then 160 V/30 min). Visualization was performed by fluorescence imaging with a Typhoon FLA 9500 instrument from GE Healthcare and the protein was visualized by standard Coomassie staining (Figure 3c).

To obtain **hCA*^{VS}** or **hCA*_coum** for MALDI-TOF analysis, the samples were dialyzed against water by using a Slide-A-Lyzer MINI dialysis unit (3500 MWCO, Thermo Scientific) and then Amicon Ultra

centrifugal filters (0.5 mL, 3 K, Merck Millipore). The products were evaporated to dryness, dissolved in water (10 μL), and analyzed by MALDI-TOF MS (for copies of mass spectra, see Figures S28 and S29 in the Supporting Information).

To use the obtained **hCA*^{VS}** in further bioconjugations with peptides, the product of the click reaction was dialyzed against water by using a Slide-A-Lyzer MINI dialysis unit (3500 MWCO, Thermo Scientific) and then Amicon Ultra centrifugal filters (0.5 mL, 3 K, Merck Millipore).

Addition of Cys-containing peptides to hCA*^{VS}

The reaction mixture (250 μL) contained **hCA*^{VS}** (2 nmol) and peptide (10 mM) in TEAA buffer (0.3 M, pH 8.6). The solution was incubated overnight at 37 °C in a thermal cycler. The samples were dialyzed against water by using a Slide-A-Lyzer MINI dialysis unit (3500 MWCO, Thermo Scientific) and then Amicon Ultra centrifugal filters (0.5 mL, 3 K, Merck Millipore). The products were evaporated to dryness, dissolved in water (10 μL), and analyzed by MALDI-TOF MS (for copies of mass spectra, see Figures S30 and S31 in the Supporting Information).

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