Supporting Information

Antibody-Drug Conjugates (ADCs) with Indolinobenzodiazepine Dimer (IGN) Payloads: DNA-Binding Mechanism of IGN Catabolites in Target Cancer Cells

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Fig. S1. Binding of IGN1-biotin, IGN2-biotin, and IGN3-biotin with digoxigenin-labeled 46-mer guanine hairpin oligonucleotide.

Fig. S2. Binding of IGN1-biotin and IGN2-biotin with purified genomic DNA in a cell-free condition.

Fig. S3. Binding of IGN1-biotin to digoxigenin-labeled 46-mer guanine hairpin oligonucleotide in the presence of competing, unlabeled inosine hairpin oligonucleotide (1-34 fold excess).

Fig. S4. Binding of IGN drugs to genomic DNA in KB cells.

Fig. S5. *In vitro* cytotoxicity of free IGN1-biotin (monoimine), IGN2-biotin (diimine), IGN3-biotin (diamine), and IGN4 (monoimine, ADC catabolite) in HSC-2 cells.

Fig. S6. Release of IGN1-biotin (monoimine) upon cleavage of cellular DNA-IGN adduct by DNase I.

Fig. S7. Release of IGN1-biotin (monoimine) upon cleavage of genomic and hairpin DNA-IGN adducts by a mixture of DNase I and MNase.

Calculations of IGN1-biotin and IGN2-biotin released from genomic DNA adducts upon DNase cleavage.

Synthesis of biotinylated IGNs.

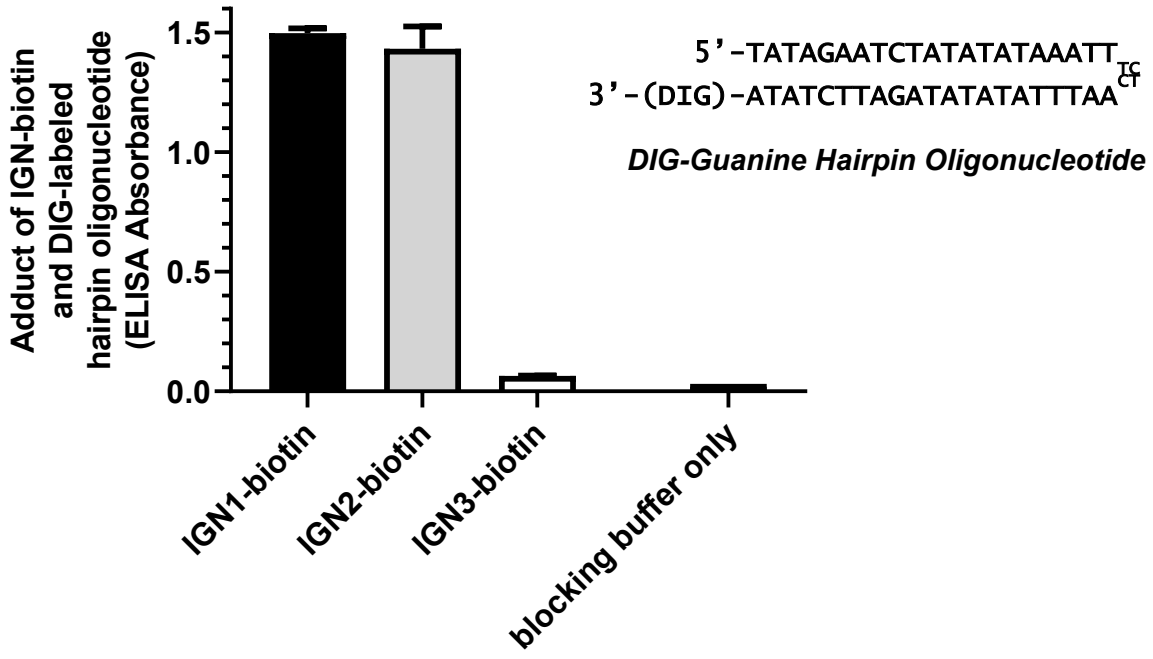


Fig. S1. Binding of IGN1-biotin, IGN2-biotin, and IGN3-biotin (50 nM) with digoxigenin (DIG)-labeled 46-mer guanine hairpin oligonucleotide (50 nM): mixtures incubated ~2 h. ELISA using coated streptavidin (IGN-biotin capture) and anti-digoxigenin antibody-horseradish peroxidase conjugate (digoxigenin-oligonucleotide detection).

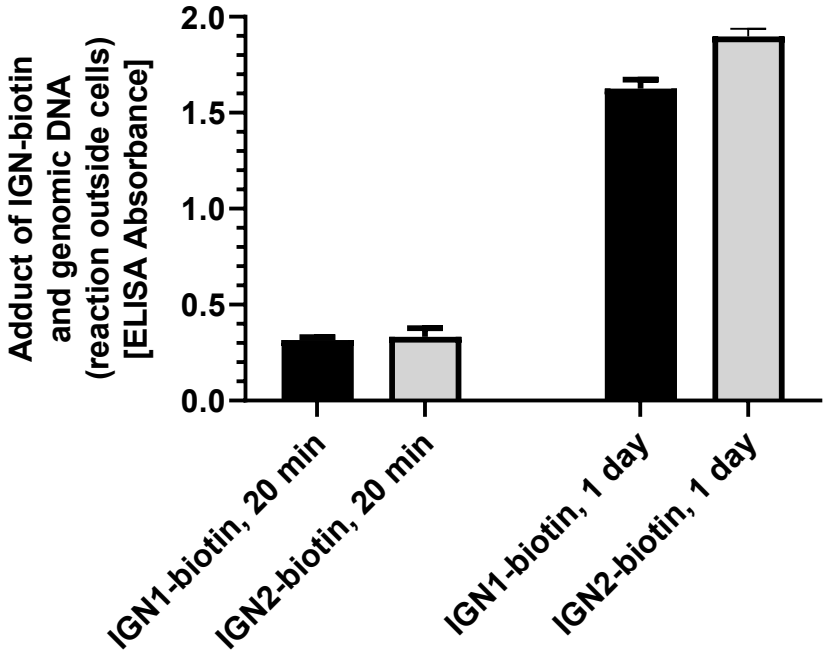


Fig. S2. Binding of IGN1-biotin and IGN2-biotin (1 μM) with purified genomic DNA: mixtures incubated in a cell-free condition for 20 min (then gel-filtered) or 1 day. ELISA using anti-DNA antibody (DNA capture) and streptavidin-horseradish peroxidase conjugate (biotin-IGN-DNA adduct detection).

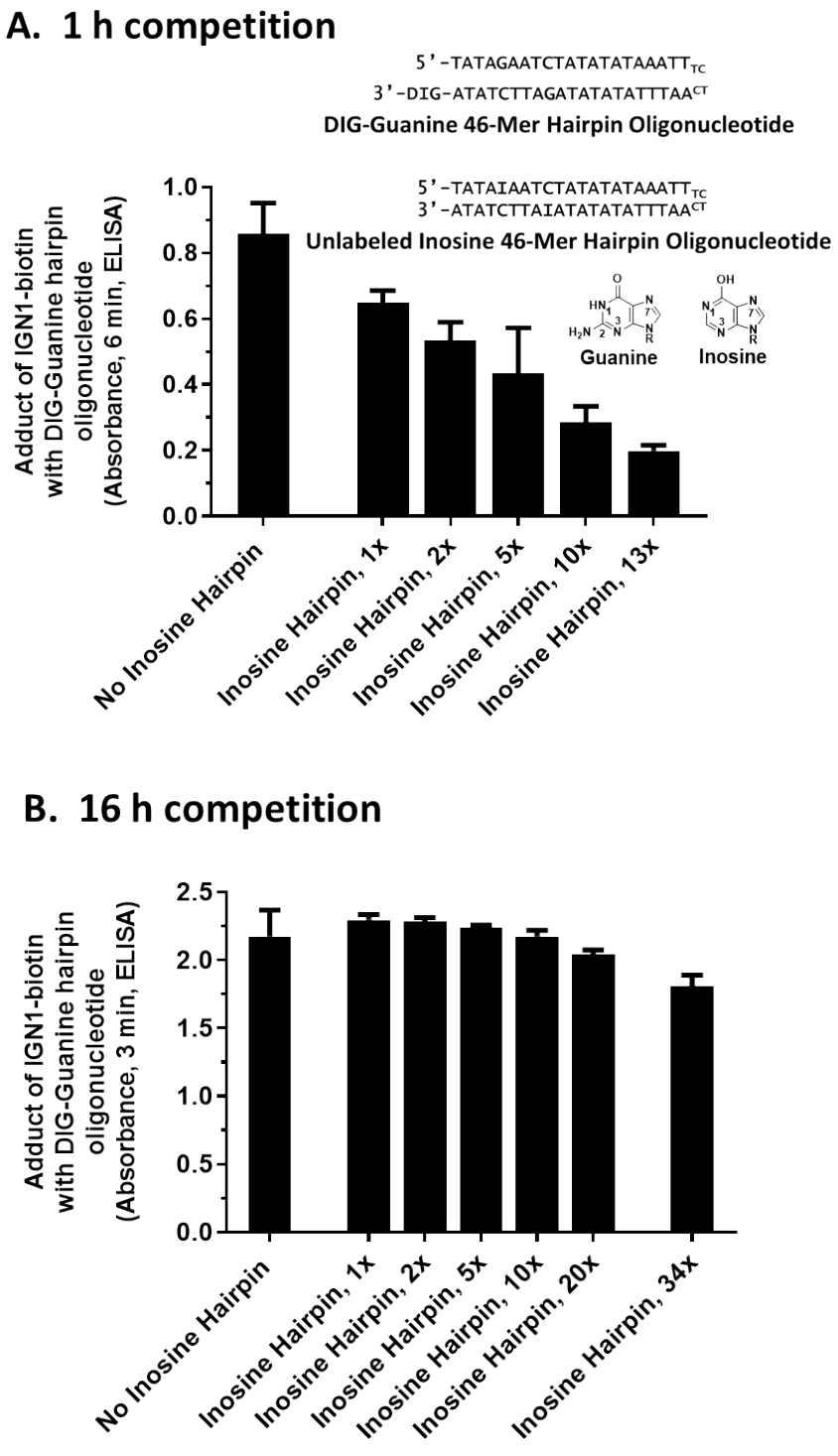


Fig. S3. Binding of IGN1-biotin (50 nM) to digoxigenin (DIG)-labeled 46-mer guanine hairpin oligonucleotide (100 nM) in the presence of unlabeled inosine hairpin oligonucleotide (100 nM - 3400 nM; 1-34 fold excess): A, short-term (1 h) and B, long-term (16 h) competition. ELISA using coated streptavidin (IGN1-biotin capture) and anti-digoxigenin antibody-horseradish peroxidase conjugate (digoxigenin-guanine hairpin oligonucleotide detection).

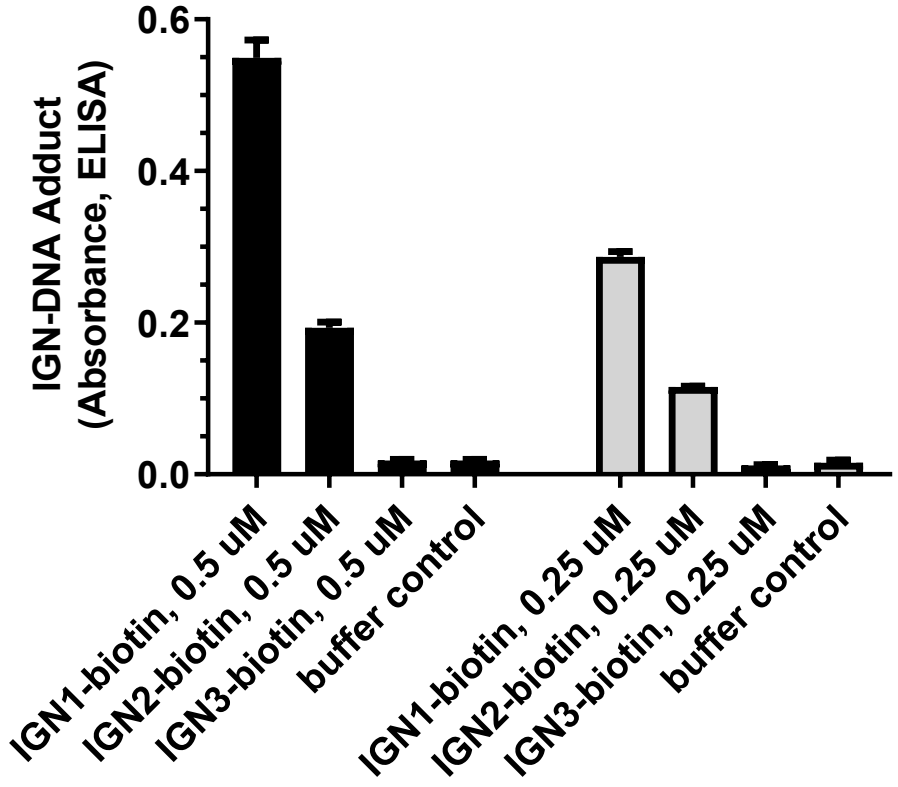
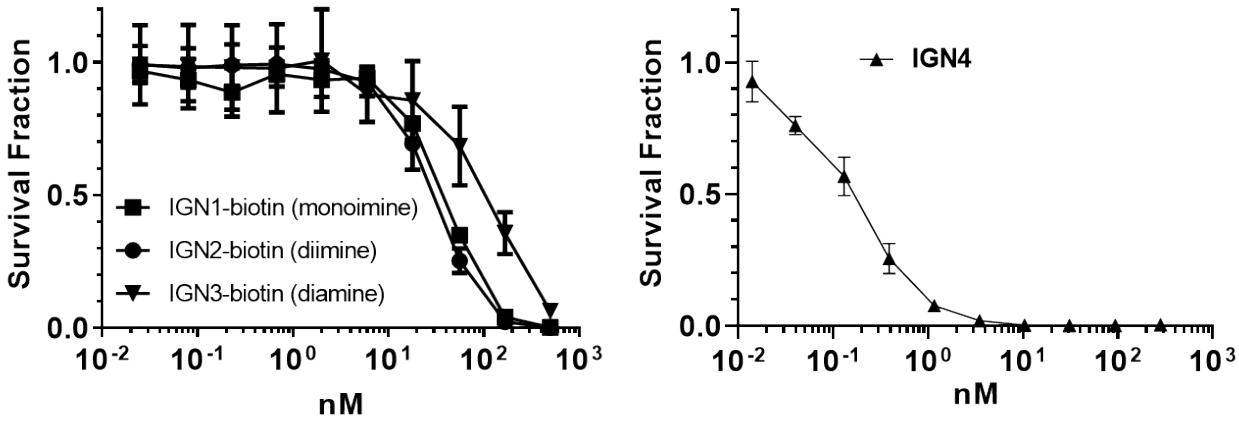


Fig. S4. Binding of IGN drugs to genomic DNA in KB cells. KB cells were treated with IGN1-biotin (monoimine), IGN2-biotin (diimine), and IGN3-biotin (diamine), each at 0.5 μM and 0.25 μM for 1 day. Genomic DNA was isolated for each treatment at 1 day, and IGN-DNA adduct analyzed by ELISA using anti-DNA antibody and streptavidin-horseradish peroxidase conjugate.



Free IGN	IC_{50} (nM), 3 day
IGN2-biotin	30
IGN1-biotin	35
IGN3-biotin	100
IGN4	0.2

Fig. S5. *In vitro* cytotoxicity of free IGN1-biotin (monoimine), IGN2-biotin (diimine), IGN3-biotin (diamine), and IGN4 (monoimine, ADC catabolite) in HSC-2 cells. HSC-2 cells were incubated with several concentrations of unconjugated IGN1-biotin, IGN2-biotin, IGN3-biotin, and IGN4 for 3 days. After 3 days, cell viabilities were measured using Cell Titer Glo reagent. Data is plotted as survival fraction (versus untreated cells).

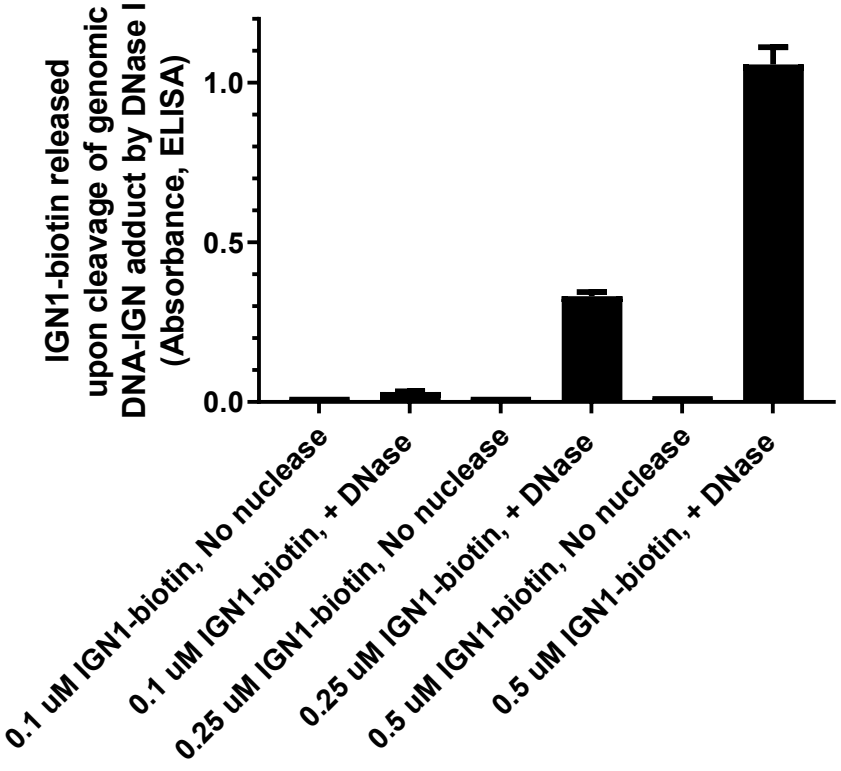


Fig. S6. Release of IGN1-biotin (monoimine) upon cleavage of cellular DNA-IGN adduct by DNase I. Genomic DNA-IGN adducts were prepared by treatment of HSC-2 cells with 0.1, 0.25, and 0.5 μM IGN1-biotin for 1 day and isolation of genomic DNA. Upon cleavage of DNA-IGN adducts by DNase I (r. t., overnight), the released IGN1-biotin was detected by ELISA using capture with coated streptavidin, followed by reaction with digoxigenin-labeled 19-mer duplex oligonucleotide and incubation with anti-digoxigenin antibody-horseradish peroxidase conjugate.

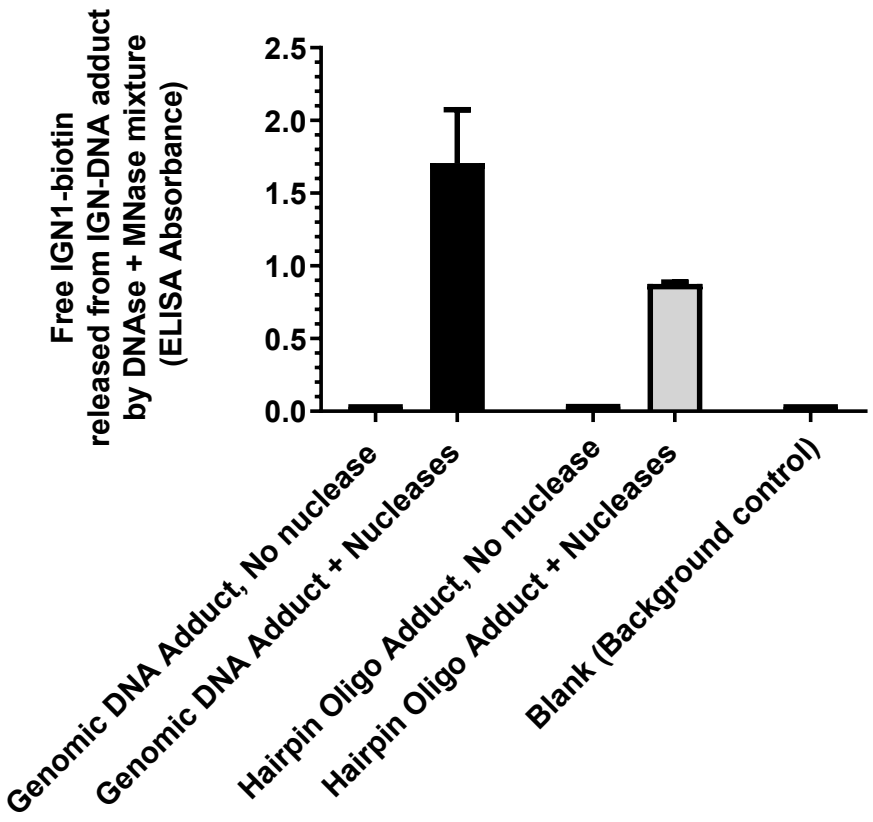


Fig. S7. Release of IGN1-biotin (monoimine) upon cleavage of genomic and hairpin DNA-IGN adducts by a mixture of DNase I and MNase ("Nucleases"). DNA-IGN adducts were prepared from purified genomic DNA of untreated HSC-2 cells (mixture of 39 μg/mL genomic DNA, in a cell-free reaction condition, with 20 μM IGN1-biotin, 1 day, r. t.), or 46-mer hairpin oligonucleotide (reaction mixture of 20 μM hairpin DNA and 25 μM IGN1-biotin, 4 days, r. t.), followed by gel-filtration using two successive columns (NAP-5, NAP-10). Upon cleavage of DNA-IGN adducts by DNase I + MNase mixture (r. t., 1 day for genomic DNA adduct; and 37 •C, 2 h for hairpin DNA adduct), the released free IGN1-biotin was detected by ELISA using immobilized streptavidin, followed by reaction with digoxigenin-labeled 19-mer duplex oligonucleotide, and incubation with anti-digoxigenin antibody-horseradish peroxidase conjugate.

Calculations of IGN1-biotin and IGN2-biotin released from genomic DNA adducts upon DNase cleavage:

HSC-2 cells (0.5 μM IGN1-biotin, IGN2-biotin treatment):
Equal levels of genomic DNA-IGN adducts of IGN1-biotin and IGN2-biotin (based on anti-DNA antibody/streptavidin-HRP ELISA) were used for DNase cleavage:
1.66 μg DNA (2.6 nmol DNA base pair) for IGN1-biotin-DNA adduct,
5.68 μg DNA (8.8 nmol DNA base pair) for IGN2-biotin-DNA adduct
(calculations based on sample per ELISA well).

IGN1-biotin released by DNase cleavage = 1 pmol,
IGN2-biotin released by DNase cleavage = 0.42 pmol
(by quantitative estimation using ELISA, with IGN1-biotin and IGN2-biotin as standards).

IGN1-biotin released per million DNA base pair = (1 pmol/2.6 nmol) x 10^6 = 380.
IGN2-biotin released per million DNA base pair = (0.42 pmol/8.8 nmol) x 10^6 = 48.

KB cells (0.5 μM IGN1-biotin, IGN2-biotin treatment):
Equal levels of genomic DNA-IGN adducts of IGN1-biotin and IGN2-biotin (based on anti-DNA antibody/streptavidin-HRP ELISA) were used for DNase cleavage:
0.99 μg DNA (1.52 nmol DNA base pair) for IGN1-biotin-DNA adduct,
1.73 μg DNA (2.66 nmol DNA base pair) for IGN2-biotin-DNA adduct
(calculations based on sample per ELISA well).

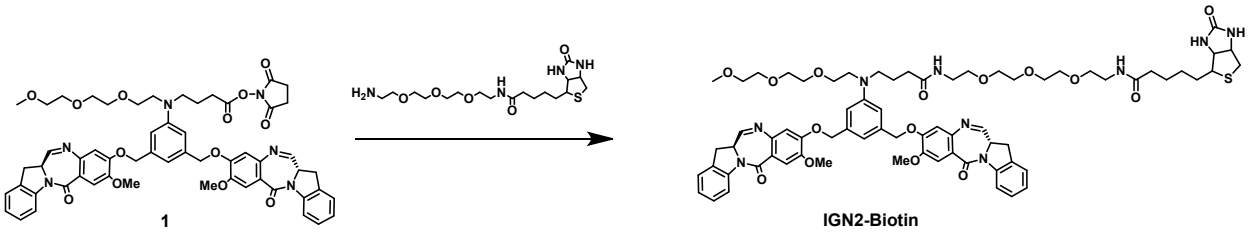
IGN1-biotin released by DNase cleavage = 0.37 pmol,
IGN2-biotin released by DNase cleavage = 0.16 pmol
(by quantitative estimation using ELISA, with IGN1-biotin and IGN2-biotin as standards).

IGN1-biotin released per million DNA base pair = (0.37 pmol/1.52 nmol) x 10^6 = 243.
IGN2-biotin released per million DNA base pair = (0.16 pmol/2.66 nmol) x 10^6 = 60.

Synthesis of biotinylated IGN dimer analogs:

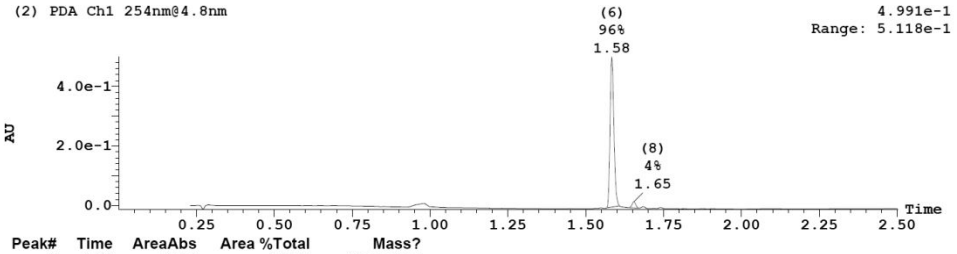
Synthesis, purification and characterization of compounds 1 and 2 are described in Miller, M. L., et. al., "A New Class of Antibody–Drug Conjugates with Potent DNA Alkylating Activity" *Molecular Cancer Therapeutics* 2016; 15, 1870-1878.

IGN2-Biotin:



Compound 1 (21 mg, 0.018 mmol) and Amine-PEG_3-Biotin (22.93 mg, 0.055 mmol; ThermoFisher Scientific) were stirred in N,N-Dimethylacetamide (609 µl) and 15 mM HEPES buffer (609 µl, pH 8.5) at room temperature for two hours under argon. The reaction mixture was directly purified by RP-HPLC (C18, Acetonitrile/Water). Fractions containing the desired product, well separated from NHS ester 1, were frozen and lyophilized to obtain compound IGN2-biotin (10.2 mg, yield = 42%) as a white solid. UPLC = 1.58 min. UPLC were acquired on a Waters, Acquity system with a single quadrupole MS Zspray™ (column: Acquity BEH C18, 2.1 x 50 mm, 1.7 µm: flow rate 0.8 mL/min, solvent A: water with 0.1% formic acid, solvent B: acetonitrile, 5 to 98% of acetonitrile over 1.8 min, and 98% acetonitrile for 0.2 min). LRMS (ESI+): Calculated for C71H87N9O15S (M + H) 1338.60, found 1339.7.

HPLC Purity for IGN2-Biotin



Peak#	Time	AreaAbs	Area %Total	Mass?
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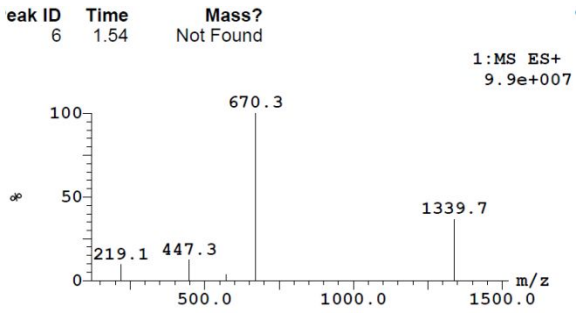
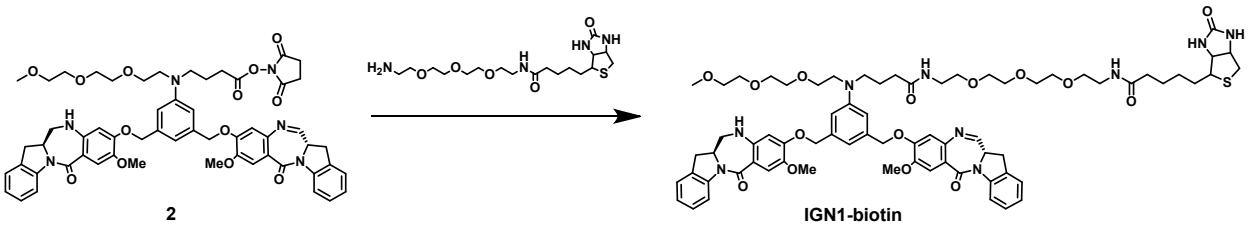
8	1.65	284	3.79	Not Found

LRMS for IGN2-Biotin

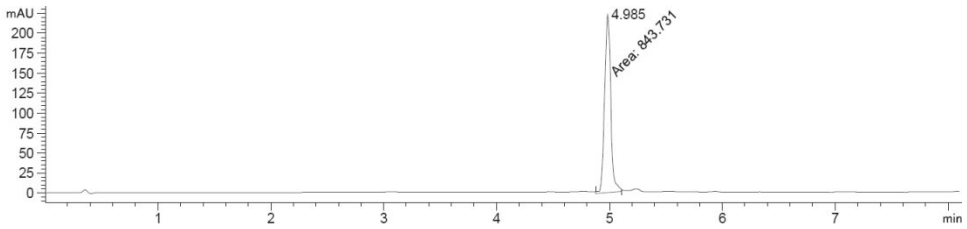


IGN1-Biotin:

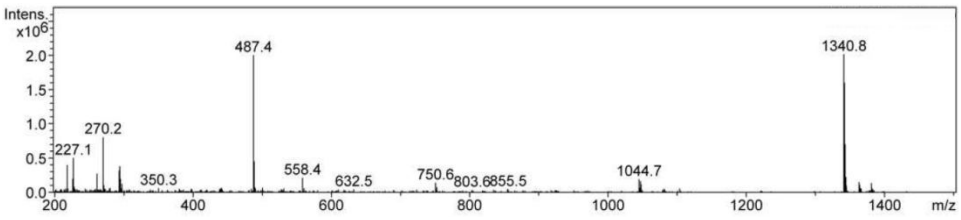


Compound 2 (15 mg, 0.014 mmol) and Amine-PEG$_3$-Biotin (18.16 mg, 0.043 mmol) were stirred in N,N-Dimethylacetamide (0.50 mL) and 15 mM HEPES buffer (0.500 mL, pH 8.5) at room temperature for five hours under argon. The reaction mixture was directly purified by RP-HPLC (C18, Acetonitrile/Water). Fractions containing product, well separated from the NHS ester 2, were frozen and lyophilized to obtain compound IGN1-biotin (12 mg, yield = 62%) as a white solid. HPLC = 4.98 min (C18; 5-98% acetonitrile gradient). LRMS (ESI+): Calculated for C71H89N9O15S (M + H) 1340.62, found 1340.8.

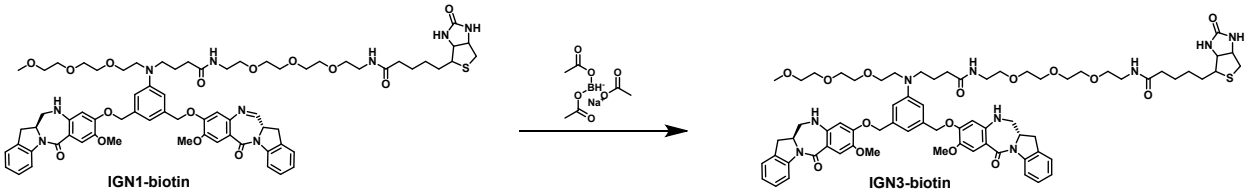
HPLC Purity for IGN1-Biotin



LRMS for IGN1-Biotin

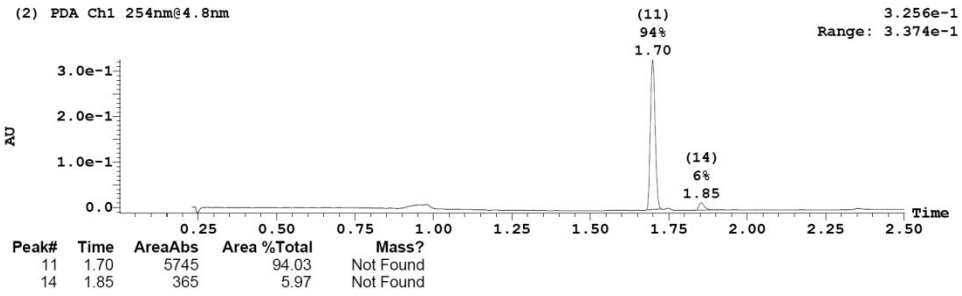


IGN3-Biotin:



To a solution of compound IGN1-biotin (5.9 mg, 4.40 µmol) in anhydrous1,2-Dichloroethane (220 µl) was added sodium triacetoxyborohydride (1.865 mg, 8.80 µmol). The reaction mixture stirred at room temperature under argon for four hours after which it was quenched with saturated ammonium chloride solution and extracted with dichloromethane. The extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered and concentrated. The crude material was purified by semi-preparative HPLC (Kromasil C18 Acetonitrile/Water). Fractions containing product were combined, frozen and lyophilized to get compound IGN3-biotin (2.5 mg, yield = 42%) as a white solid. UPLC = 1.70 min. LRMS (ESI+): Calculated for C71H91N9O15S (M + H) 1342.64, found 1343.2.

HPLC Purity for IGN3-Biotin



Peak#	Time	AreaAbs	Area %Total	Mass?
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14	1.85	365	5.97	Not Found

LRMS for IGN3-Biotin