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Biolabeling with 2,4-Dichlorophenoxyacetic Acid Derivatives: The 2,4-D Tag

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Many bioanalytic and diagnostic procedures rely on labels with which the molecule of interest can be tracked in or discriminated from accompanying like substances. Herein, we describe a new labeling and detection system based on derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D) and anti-2,4-D antibodies. The 2,4-D system is highly sensitive with a K_D of 7×10^{-11} M for the hapten−antibody pair, can be used on a large variety of biomolecules such as proteins, peptides, carbohydrates, and nucleic acids, is not hampered by endogenous backgrounds because 2,4-D is a xenobiotic, and is robust because 2,4-D is a very stable compound that withstands the conditions of most reactions usually performed on biomolecules. With this unique blend of properties, the 2,4-D system compares favorably with its rivals digoxigenin (DIG)/anti-DIG and biotin/(strept)avidin and provides an interesting and powerful tool in biomolecular labeling.

In the course of many analytical and diagnostic procedures, the sensitive and specific detection of biomolecules, such as peptides, proteins, oligosaccharides, or DNA, is of critical importance. Hence, reagents and methodologies that are suitable for the labeling, detection, and quantification of target-molecules present in complex biological samples are urgently required. In recent years, numerous nonradioactive labeling methods have been developed. Target molecules can be equipped either with "indirect labels" (e.g., biotin, digoxigenin) or with "direct labels" (fluorophores or luminophores). While direct labels allow immediate visualization of derivatized target molecules, indirect labels require the use of secondary detection reagents. Sensitivity of detection of indirect labels is often superior as signal amplifying detection reagents can be used and self-quenching, a common problem of direct fluorophore labels, can be avoided in indirect setups.

Biotin/(strept)avidin and digoxigenin (DIG)/anti-DIG are the most commonly used indirect labeling systems in bioanalytical applications.[1−4] Nevertheless, these systems have several drawbacks that limit their use, for example, the endogenous occurrence of biotin in various biological sources (serum, plasma, tissues, cells, organelles, e.g., mitochondria),[4,5] the uptake of biotin or DIG by epithelial transporters,[6,7] or the metabolic modification of DIG by intestinal bacteria.[8] Because multiple labeling is frequently required in modern analytical and diagnostic procedures[9,10] it is necessary to improve the bioanalytical toolbox with new labeling and detection reagents that are versatile and robust and can be applied in combination with established systems.

Here, we describe a novel indirect labeling/detection system based on 2,4-dichlorophenoxyacetic acid (2,4-D, **(1)**; see Figure S-1 in the Supporting Information) and anti-2,4-D antibodies. 2,4-D has been used as a herbicide since the 1950s,[11] and antibodies against 2,4-D have been developed[12,13] to determine the 2,4-D burden in the environment, e.g., in drinking water, food or body fluids.[14,15] In the competition-type immunoassays used for this purpose, the 2,4-D-specific antibodies displayed a satisfactory

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sensitivity, with lower detection limits of 1×10^{-7} g/L 2,4-D.[16] However, the use of 2,4-D/anti-2,4-D as an indirect labeling system has not been pursued since any derivatization of substrates with 2,4-D had to be performed in situ in the presence of condensing agents and organic solvents.[17]

Our new 2,4-D derivatives allow the straightforward labeling of numerous biological substrates, such as peptides, proteins, and DNA, under physiological conditions. Moreover, a modification of the 2,4-D derivatives with aliphatic spacers improves their binding toward the 2,4-D-specific antibodies resulting in a greatly increased sensitivity of detection. The 2,4-D-based system, therefore, represents a valuable addition to the field of indirect labeling systems, comparing favorably with established systems and facilitating multiple labeling applications.

EXPERIMENTAL SECTION

Additional Methods. General methods, materials, syntheses, and analyses of the 2,4-D-based labeling derivatives are described in the Supporting Information (Supplementary Methods and Figures S-1−S-4).

Determination of Hydrolysis Half-Lives of Active Esters. The deuterolysis of active esters in phosphate-buffered deuterium oxide (D_2O) was determined by ^{1}H NMR spectroscopy. Stock solutions of active esters **(2)**, **(5)**, **(6)**, digoxigenin (digoxigenin-C6-*N*-hydroxysuccinimide (DIG-C6-NHS)) and biotin (biotin-*N*-hydroxy-sulfosuccinimide (biotin-NHSS), biotin-C6-NHSS, and biotin-C11-NHS) (each 50 mM) were prepared in anhydrous DMSO-d_6. Deuterolysis was initiated by adding 10 μL of these solutions to 490 μL of 50 mM deuterated phosphate-buffer, pD 7.2, containing 0.1 mg/mL 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (TMSPS), and monitored with an Avance DRX-600 system (Bruker BioSpin, Rheinstetten, Germany) at 600 MHz. ^{1}H-spectra were recorded after 6−12, 20, 40, 60, 120, 240, 360, and 1000−1600 min (overnight). Data were acquired and processed using the X-WIN-NMR software (V 2.5) (Bruker BioSpin). Discrete peaks obtained at the various points in time and originating from the (sulfo-)succinimidyl moiety and from the aliphatic spacer were integrated and standardized in relation to TMSPS. From the peak integrals, the percentage of nonhydrolyzed active ester derivative could be calculated (for details see the Supplementary Methods in the Supporting Information).

Labeling of Biomolecules with Reactive Derivatives of 2,4-D, Biotin, and DIG and Detection of Labeled Compounds. Stock solutions of 2,4-D active esters **(2)**, **(5)**, **(6)** or respective active esters of digoxigenin (DIG-C6-NHS) or biotin (biotin-NHSS, biotin-C6-NHSS or biotin-C11-NHS) were prepared freshly in anhydrous DMSO for each labeling experiment.

For labeling of ovalbumin, 100 nmol of the protein (2 μmol amino functions) were reacted with 1.5 μmol of active ester in 100 mM sodium tetraborate, pH 8.2, containing no more than 5% (v/v) DMSO. Labeling reactions were terminated by adding 1.5 mmol glycine. Serially diluted labeled protein was applied onto nitrocellulose membranes (16000−3.12 pg conjugate/dot). After washing with Dulbecco's phospate-buffered saline (D-PBS: 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) and blocking with 1% (w/v) casein in D-PBS (casein-PBS), the labeled proteins were detected with either monoclonal antibodies directed against 2,4-D or DIG followed by AlexaFluor680-labeled goat anti-mouse IgG or with AlexaFluor680-labeled streptavidin. Fluorescence was quantitated with an Odyssey infrared imaging system using the Odyssey software (V2.1) (LI-COR Biosciences, Bad Homburg, Germany).

For labeling of insulin, 35 nmol of the protein (105 nmol amino functions) was reacted with 350 nmol active ester in 50 mM phosphate buffer, pH 7.2, containing no more than 2% (v/v) DMSO. Labeling reactions were terminated by addition of 1 mmol of glycine. The molecular mass and heterogeneity of the samples was determined by MALDI-TOF-MS, and the mean degree of labeling was calculated as previously described.[23] Serially diluted labeled protein was applied onto preactivated polyvinylidene difluoride (PVDF)-membrane-bottomed polystyrene filterplates (1000−0.12 ng conjugate/well) and incubated for 30 min. After washing with D-PBS containing 0.1% (v/v) Tween 20 and D-PBS followed by blocking with 2% (w/v) casein hydrolysate in D-PBS, the labeled protein was detected as above except that horseradish peroxidase (HRP)-labeled detection reagents in combination with a tetramethylbenzidine-based substrate[24] were used for visualization.

Glycoproteins were labeled in situ on membranes. Serially diluted porcine mucin was applied to nitrocellulose (2000−1 pg glycoprotein/dot) and the carbohydrate moieties were oxidized with sodium meta-periodate. After washing with D-PBS, membranes were incubated in 2.5 μM hydrazide **(7)**, **(10)**, **(11)** (Figure 2) or the respective hydrazide of digoxigenin or biotin, washed again, blocked, and processed with the monoclonal antibodies and the AlexaFluor680-labeled detection reagents as described above (for details see the Supplementary Methods in the Supporting Information).

Labeling of Peptides with 2,4-D Derivatives and Detection Thereof. Fmoc-protected lysine derivatives **(19)**, **(20)**, and **(21)** were synthesized by reacting the active esters **(2)**, **(8)**, or **(9)** with Fmoc-L-lysine-OH. Peptide ASQLDYKMTDAGE (N- to C-terminus) was solid-phase-synthesized by standard Fmoc-chemistry. Lysine-*N*-ε-(biotin) was incorporated during peptide synthesis. Peptides were labeled at their amino terminus with 2,4-D by reacting 2,4-D compound **(1)**, **(3)**, **(4)**, **(12)**, **(13)**, or **(14)** with the completed peptide chain after removal of the aminoterminal Fmoc protection group. A carboxyterminal 2,4-D label was introduced by the use of 2,4-D-lysine derivative **(19)**, **(20)**, or **(21)** as first amino acid in the peptide synthesis process.

To determine the limit of detection (LOD), 96 well microplates were coated with 18.75 ng/well anti-2,4-D antibody (clone F6/C10, E2/G2 or 4B7), washed with D-PBS, blocked with casein-PBS, and washed again. Peptides serially diluted in casein-PBS were applied (25.0 pmol−11.9 amol conjugate/well) and incubated for 2.5 h at RT, and the plates were washed again. To follow the association reaction, coated plates were incubated for 5, 10, 20, 40, or 80 min with 167 nM peptide and washed. For the dissociation reaction, plates were incubated for 2.5 h with 167 nM

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peptide, washed, refilled with casein-PBS, incubated for 5, 10, 20, or 80 min, and washed. In all experiments, bound peptide was then quantitated with HRP-streptavidin as described above (for details see the Supplementary Methods in the Supporting Information).

Data Analysis and Statistics. Limits of detection (LOD) were determined by *t*-statistics (95% confidence interval).[25] For statistical analyses, LOD data were transformed logarithmically, and an outlier analysis (extreme studentized deviate method) and one-way ANOVA with Bonferroni post hoc test were performed. LOD data were then back-transformed from the logarithmic state and are presented as geometric mean and 95% confidence interval. Hydrolysis half-lives of active esters were calculated by nonlinear regression (one-phase exponential decay logistic function). Maximum optical density at 450 nm (MaxOD) and steady-state dissociation constant K_D (mean ± SD) were determined by nonlinear regression analysis (one-site binding logistic function). Variation in and differences of hydrolysis half-lives, MaxOD or K_D were analyzed by an outlier analysis (extreme studentized deviate method) and one-way ANOVA with Bonferroni post hoc test. Statistical and regression analyses were done using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). For all statistical analyses, a probability of $P < 0.05$ was considered significant.

RESULTS

To achieve a broad versatility of the new labeling system, active esters, hydrazides, and amino acid- and nucleotide-derivatives of 2,4-D were devised and synthesized (detailed synthesis schemes and structural formulas in Figures S-1−S-4 in the Supporting Information). These compounds, along with equivalent DIG or biotin derivatives, were then used for labeling of proteins, glycoproteins, peptides, and DNA, and the detection performance of the three systems was compared in various experimental setups.

Labeling of Proteins via Primary Amines. Labeling groups are commonly introduced in proteins by reacting active ester derivatives of the label with the protein's surface-exposed primary amino functions. We, therefore, conceived appropriate active esters of a variety of 2,4-D derivatives carrying either no aliphatic spacer moieties or aliphatic spacer moieties of different length (Figure 1A and Figure S-1 in the Supporting Information). 2,4-D **(1)** was converted into its *N*-hydroxysuccinimide (NHS)-ester **(2)** by reacting **(1)** with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and NHS. Spacered 2,4-D derivatives were synthesized using aminohexanoic (C6) and aminoundecanoic (C11) acid. Since these alkyl amino acids were hardly soluble in organic solvents that are suited for coupling, they were first converted into their tetrabutyl ammonium salts before being reacted with **(2)** to yield the respective 2,4-D derivatives **(3)** and **(4)**. 2,4-D has low solubility in water (0.7 g/L), and the aliphatic C6- or C11-spacers further decrease its solubility. Hence, we replaced NHS by *N*-hydroxy-sulfosuccinimide (NHSS) to introduce the amine reactive functional group along with a hydrophilic sulfonic acid residue. NHSS-esters **(5)** and **(6)** were synthesized by reacting carboxylic acids **(3)** or **(4)** with DCC and NHSS. The charged NHSS-moiety renders the respective 2,4-D derivatives suitable for protein labeling under physiological conditions.

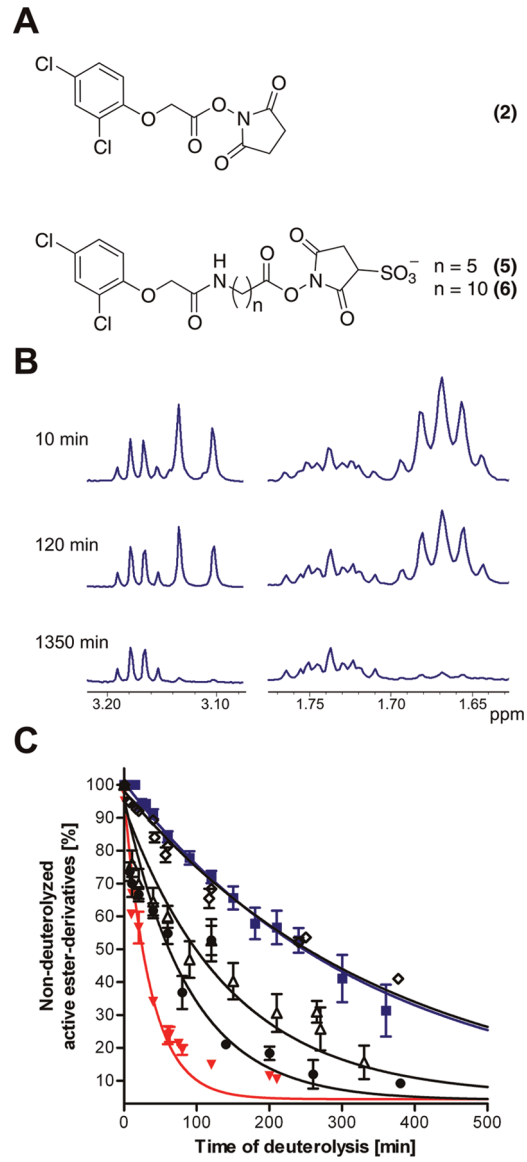

Figure 1. Stability of 2,4-D active ester derivatives in aqueous solutions. (A) *N*-hydroxysuccinimidyl-(NHS)-ester **(2)** or *N*-hydroxysulfosuccinimidyl-(NHSS)-ester derivatives **(5)** and **(6)** of 2,4-dichlorophenoxyacetic acid were synthesized. Derivatives **(5)** and **(6)** had been equipped with aliphatic 6-aminohexanoyl (C6) or 11-aminoundecanoyl (C11) spacers, respectively. (B, C) The deuterolysis of **(2)**, **(5)**, and **(6)** was measured via ^{1}H NMR spectroscopy in 50 mM phosphate buffer, pD 7.2, containing 2% (v/v) DMSO-d_6, and was compared with deuterolyses of active ester derivatives of digoxigenin (DIG) or biotin. (B) Exemplarily, three sections of ^{1}H NMR spectra of **(5)** after 10, 120, and 1350 min at room temperature are shown. Signals which were altered in intensity or chemical shift due to deuterolysis and which were not superimposed by signals of other nuclei were integrated and put in relation to the internal standard 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt. The deuterolysis of each active ester derivative was measured in 2 to 3 independent experiments. (C) The concentration of the nondeuterolyzed active ester derivatives was calculated from the change of peak integrals and was plotted versus time (▼, **(2)**; ■, **(5)**; ◇, DIG-C6-NHS; •, biotin-NHSS; △, biotin-C6-NHSS). Deuterolysis half-lives as well as the 95% confidence intervals were determined by nonlinear regression analyses (see Table S-1 in the Supporting Information). Deuterolysis half-lives of **(5)** (231 min; mean) and DIG-C6-NHS (248 min, mean) are significantly above deuterolysis half-lives of all other active esters (one-way ANOVA, Bonferroni post hoc test, $P < 0.001$) and do not differ from each other ($P > 0.05$).

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Table 1. Limits of Detection (LOD) of Labeled Protein Conjugates[a]

label	primary antibody (clone)	spacer	limit of detection [pg] of labeled protein: ovalbumin	insulin	mucin
2,4-D	E2/G2	–	171.0[b]	179215.9[b]	125.0
		C6	322.8[b]	1139.2[c]	27.9[d]
		C11	582.1[b]	111.9[e]	42.8[f]
	E4/C2	–	171.0[b]	8277.5[b]	152.4
		C6	80.7[b]	44.9[c]	14.2[d]
		C11	292.4[b]	11.7[e]	19.4[d]
	F6/C10	–	182.0[b]	170215.9[b]	111.4
		C6	192.8[b]	1139.2[c]	3.3[g]
		C11	250.0[b]	111.9[e]	15.9[h]
	B7	–	278.6[b]	157398.3[b]	80.7[i]
		C6	292.4[b]	1550.2[c]	9.8[h]
		C11	479.7[b]	122.1[e]	15.6[h]
DIG	1.71.256	–	n.a.	n.a.	n.a.
		C6	120.5[b]	19678.9[j]	76.2[i]
		C11	n.a.	n.a.	n.a.
biotin		–	174.6[b]	57809.6[b]	220.8
		C6	179.5[b]	28906.8[b]	46.5[f]
		C11	35318.3	1078946.7	125.0

[a] Ovalbumin and insulin were labeled with active ester derivatives **(2)**, **(5)**, or **(6)** of 2,4-D (Figure 1) or with comparable derivatives of DIG or biotin and were immobilized on nitrocellulose or PVDF. Oxidized mucin that had been immobilized on nitrocellulose was labeled with hydrazide derivatives **(7)**, **(10)**, or **(11)** of 2,4-D (Figure 2) or with comparable derivatives of DIG or biotin. 2,4-D and DIG labels were detected by specific primary antibodies and AlexaFluor680-labeled (ovalbumin, mucin conjugates) or horseradish peroxidase-(HRP)-labeled (insulin conjugates) secondary antibodies; biotin labels were visualized with streptavidin-AlexaFluor680 (ovalbumin, mucin conjugates) (numbers of experiments (N_{exp}): (7−8) or HRP-streptavidin (insulin conjugates) (N_{exp}: 8−12). n.a.: no corresponding labeling reagent available. (95% confidence intervals of LOD are shown in Table S-2 in the Supporting Information.) [b] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with C11-spacered biotin ($P < 0.001$). [c] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with C6-spacered DIG, biotin, C6-spacered biotin, C11-spacered biotin, and 2,4-D ($P < 0.001$). [d] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with C6-spacered DIG, biotin, C11-spacered biotin, and 2,4-D ($P < 0.05$). [e] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with C6-spacered DIG, biotin, C6-spacered biotin, C11-spacered biotin, 2,4-D, and C6-spacered 2,4-D ($P < 0.01$). [f] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with biotin and C11-spacered biotin ($P < 0.01$). [g] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with all other conjugates ($P < 0.001$). [h] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with C6-spacered DIG, biotin, C6-spacered biotin, C11-spacered biotin, and 2,4-D ($P < 0.05$). [i] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with biotin ($P < 0.01$). [j] A significantly lower (one-way ANOVA with Bonferroni post hoc test) LOD as compared to protein labeled with biotin and C11-spacered biotin ($P < 0.01$).

For labeling of model proteins, 2,4-D-active esters **(2)**, **(5)**, and **(6)** as well as comparable active esters of biotin and DIG were used. Since not all of these labeling reagents were available as water-soluble NHSS-esters, the labeling reactions were performed at neutral to basic pH in the presence of <5% organic solvent. To find out whether hydrolysis of the active esters under such conditions might diminish the labeling efficiency, their stability was measured with a nuclear magnetic resonance (NMR)-based method that was developed for this task and enabled direct surveillance of the deuterolysis reaction in deuterated buffer (Figure 1B,C). The shortest half-life of approximately 25 min was determined for the nonspacered 2,4-D derivative **(2)**, all other active esters withstood deuterolysis considerably longer with half-lives ranging from 1 h up to 4 h (see Table S-1 in the Supporting Information for exact deuterolysis half-lives). The deuterolysis of the C11-spacered compounds could not be determined as these substances precipitated under the experimental conditions. Although the various active esters differ in their stability, which may influence their labeling efficacy, all measured half-lives appear to be sufficiently high to provide enough reactive agent for labeling in aqueous solutions.

To investigate the protein labeling characteristics of the 2,4-D-active esters and their competitors, we chose ovalbumin and insulin as model substrates. These proteins have similar isoelectric points (5.01 vs 5.28) but differ in molecular size (45 vs 5.7 kDa) and number of lysines per molecule (20 vs 1).

Ovalbumin was conjugated to 2,4-D, biotin, or DIG using the respective active esters described above. The conjugates were immobilized on nitrocellulose membranes and detected with fluorophore-labeled detection reagents (see Figure S-5 in the Supporting Information). During the labeling reaction, the C11-spacered biotin, but not the C11-spacered 2,4-D derivative **(6)**, precipitated, indicating that the labeling may not have been as effective as for the other compounds used. Indeed, the limit of detection (LOD) of all other conjugates was significantly lower than that of the biotin-C11-ovalbumin conjugate (ANOVA, Bonferroni post hoc test, $P < 0.001$) (Table 1). All other conjugates showed similar LOD, the lowest value, approximately 2 fmol labeled ovalbumin conjugate, was obtained with derivative **(5)** and anti-2,4-D antibody clone E4/C2. The various aliphatic spacers in the 2,4-D labeling compounds had no discernible effect on the detectability of ovalbumin. A determination of the degree of labeling of the ovalbumin conjugates by mass spectrometry was not possible due to the high molecular mass and the heterogeneity of the ovalbumin preparation.

Insulin was labeled analogously, and here, the mean degree of labeling (MDOL) could be determined to be approximately 1.5 mol label/mol insulin. Only **(2)**, **(6)**, and biotin-C11-NHS generated lower MDOL in the range of 0.6−0.1 mol label/mol insulin (see Figure S-6 in the Supporting Information). In variation of the experimental setup, insulin conjugates were immobilized on PVDF filterplates and visualized with a peroxidase-based detection

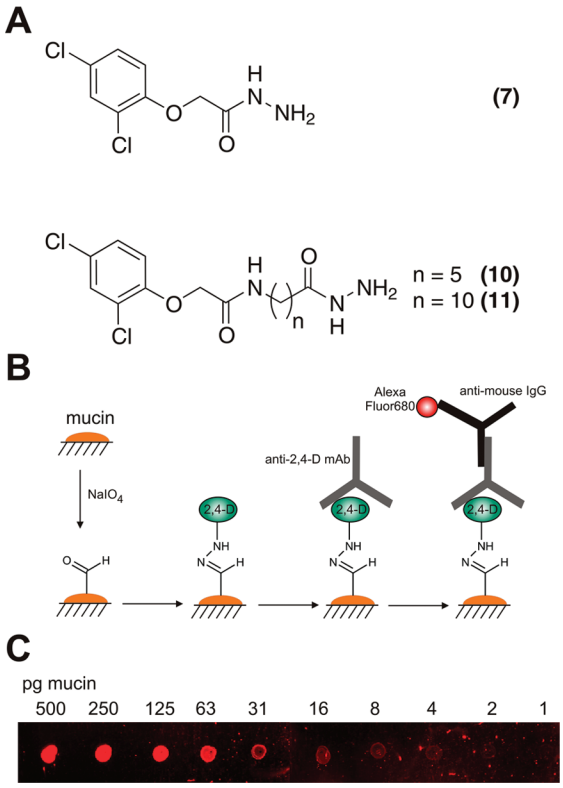

Figure 2. Labeling of glycoprotein with hydrazide derivatives. (A) Hydrazide derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D), which contained either no spacer, **(7)**, an aminohexanoyl (C6) spacer, **(10)**, or an aminoundecanoyl (C11) spacer, **(11)**, were synthesized and used for the labeling of oxidized carbohydrates of the glycoprotein mucin under physiological conditions. (B) Mucin was immobilized on nitrocellulose, oxidized with $NaIO_4$, and labeled with hydrazide derivatives **(7)**, **(10)**, or **(11)**, or comparable hydrazide-derivatives of digoxigenin (DIG) or biotin. 2,4-D and DIG labels were detected by specific primary antibodies and AlexaFluor680-labeled secondary antibody whereas biotin labels were visualized with streptavidin-AlexaFluor680 (N_{exp}: 7). (C) A representative dot blot with anti-2,4-D antibody clone E4/C2 and mucin labeled with **(10)** is shown.

system (see Figure S-5 in the Supporting Information). In contrast to ovalbumin, the use of spacered 2,4-D derivatives increased the sensitivity of insulin detection by a factor of up to 1500 compared to the unspacered 2,4-D derivative (Table 1). Thus, as little as 2 fmol of insulin labeled with 2,4-D derivative **(6)** could be detected with anti-2,4-D antibody clone E4/C2. Thereby, the 2,4-D detection system by far outperformed its competitors DIG and biotin which required at least 3 pmol of labeled protein to generate a signal.

Labeling of Glycoproteins via Oligosaccharides. For tagging of carbohydrate structures, we devised a specific labeling strategy where 2,4-D hydrazides are reacted with the oxidized carbohydrate moieties of a glycoprotein. 2,4-D hydrazide **(7)** (Figure 2A) was synthesized by reacting the acid chloride of **(1)** with hydrazine hydrate. The alkyl-spacered 2,4-D hydrazides **(10)** and **(11)** (Figure 2A) were generated by first converting 2,4-D compounds **(3)** and **(4)** into their corresponding NHS-esters **(8)** and **(9)** which were subsequently reacted with hydrazine hydrate (see Figure S-2 in the Supporting Information).

The highly glycosylated protein mucin (>200 kDa, 85% carbohydrates) was chosen as substrate molecule for the labeling reaction, which was carried out in situ after immobilizing the glycoprotein on nitrocellulose membranes. The cis-diol functions of the carbohydrate moieties of the immobilized mucin were oxidized, thereby generating aldehyde functions which then were reacted with hydrazides **(7)**, **(10)**, or **(11)** or with corresponding hydrazides of biotin or DIG. The conjugates were detected with appropriate fluorophore-labeled detection reagents (Figure 2B,C). The sensitivity of detection was increased up to 35-fold by the use of alkyl-spacered 2,4-D hydrazides **(10)** or **(11)**, as compared to nonspacered 2,4-D hydrazide **(7)**. Applying 2,4-D hydrazide **(10)**, the LOD was 15−23-fold lower than after biotin or DIG labeling and allowed the detection of 3 pg (~15 amol) of labeled mucin (Table 1).

Effect of Polyethyleneglycol Substructures in Labeling Compounds. In an attempt to further improve the performance of the 2,4-D derivatives by increasing their solubility, polyethylene glycol (PEG_{11}) moieties were introduced in 2,4-D compounds **(1)**, **(3)**, and **(4)** in a solid-phase synthesis such that the aliphatic moiety remained adjacent to 2,4-D. The 2,4-D-PEG_{11} compounds **(12)**, **(13)**, and **(14)** (see Figure S-3 in the Supporting Information) were converted into their corresponding NHS-esters **(15)**, **(16)**, and **(17)** which then were used to label and detect insulin as described above. Albeit the PEG_{11} moieties brought about an excellent solubility in aqueous solutions and improved the sensitivity of detection obtained after labeling with **(2)**, the sensitivity of detection obtained with the spacered NHSS-esters **(5)** and **(6)** could not be improved further by introducing PEG_{11} (see Figure S-7 in the Supporting Information).

Double Labeling of Peptides and Evaluation of Binding between 2,4-D and Anti-2,4-D Antibodies. A double-labeled 13 mer peptide was used to determine the binding parameters between various 2,4-D derivatives and anti-2,4-D antibodies in situ. A biotin label was introduced during solid-phase peptide synthesis via incorporation of lysine-*N*-ε-(biotin). For the 2,4-D label, peptides were either derivatized at their amino terminus with 2,4-D compound **(1)**, **(3)**, **(4)**, **(12)**, **(13)**, or **(14)** (see Figures S-1−S-4 in the Supporting Information) or at their carboxy terminus with 2,4-D-lysine derivative **(19)**, **(20)**, or **(21)** (Figure 3A). In an ELISA-type setup using anti-2,4-D antibodies to capture the peptides and HRP-labeled streptavidin for detection, the LOD for 2,4-D-labeled peptides without aliphatic spacer ranged from 0.7 to 25 fmol. The aliphatic spacers, but not the PEG-moieties, lowered the LOD 50−65-fold and allowed the detection of about 100 amol of 2,4-D-labeled peptide (Table 2). In the same system, the dissociation constants for the complex between 2,4-D derivatives and anti-2,4-D antibodies were determined (Table 2). Here again, a beneficial effect of the aliphatic spacers on the affinity was observed, the respective 2,4-D derivatives showing a K_D in the range of 100 pM, while nonspacered variants showed an approximately 10-fold higher dissociation constant. This effect was mainly due to a decreased dissociation rate of the spacered compared to the nonspacered 2,4-D derivatives (Figure 3B) and gave rise to an up to 8-fold increase of the MaxOD under steady-state conditions (Table 2).

Reversible Labeling of Primary Amines. The possibility to reversibly label a biomolecule is of particular interest for affinity purification purposes. To include this option in the 2,4-D toolbox, 2,4-D dimedone **(18)** was synthesized which can be attached to

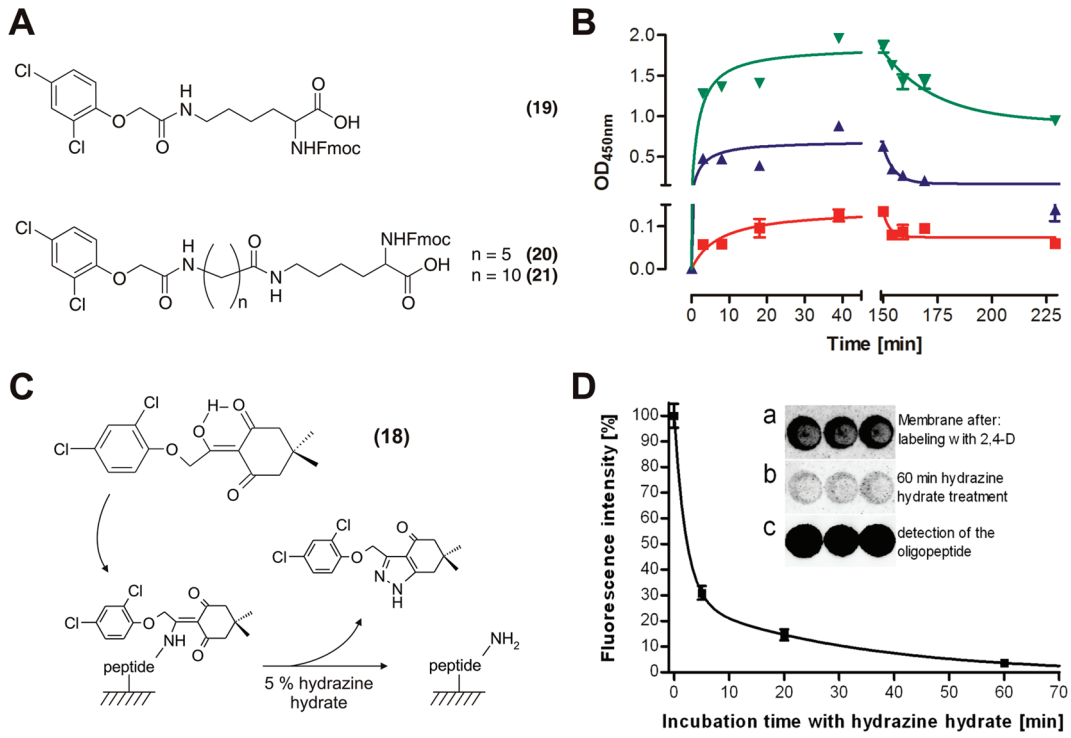

Figure 3. 2,4-D labeling of peptides. (A) Fmoc-protected lysines were synthesized equipped with 2,4-dichlorophenoxyacetyl (2,4-D) moieties on their side chains. The 2,4-D moieties either carried no spacer **(19)**, an aminohexanoyl (C6) **(20)** spacer, or an aminoundecanoyl (C11) **(21)** spacer. (B) Peptides (sequence (N- to C-terminus): ASQLDYKMTDAGE) were synthesized in double-tagged form with 2,4-D derivatives **(1)**, **(3)**, **(4)**, **(12)**, **(13)**, **(14)**, **(19)**, **(20)**, or **(21)** (see Figures S-1−S-4 in the Supporting Information) and lysine-*N*-ε-(biotin), respectively (Table 2). The association and dissociation behaviors of the peptides were determined by ELISA (N_{exp}: 3). Representative association and dissociation profiles of peptides to anti-2,4-D antibody clone F6/C10 are shown (peptide labeled N-terminally with 2,4-D derivatives **(1)** (■), **(3)** (▲), or **(4)** (▼)). (C, D) For reversible 2,4-D labeling, 2,4-D dimedone **(18)** was synthesized. Membrane anchored peptides (sequence (N- to C-terminus): GSIGAASMEFCFDCF) derived from ovalbumin were N-terminally labeled with **(18)** and the bound 2,4-D label was detected with biotinylated anti-2,4-D-antibody clone E4/C2 and AlexaFluor680-labeled streptavidin. The fluorescence signals were quantitated ((D), inset a) and **(18)** was cleaved off by incubation of the membrane with 5% (v/v) hydrazine hydrate. (D) The efficacy of deprotection of **(18)** was monitored via measurement of the remaining fluorescence (mean ± SD) at various points in time (**(D)**, inset b). When the 2,4-D tag was completely removed from the peptides, the peptides were N-terminally acetylated and detected with monoclonal anti-ovalbumin antibody and AlexaFluor680-labeled secondary antibody ((D), inset c) (N_{exp}: 6).

Table 2. Binding Characteristics of 2,4-D-Labeled Peptides[a]

labeled peptide	2,4-D derivative used for labeling	MaxOD mean ± SD	K_D [pM] mean ± SD	LOD of labeled peptide [amol] geometric mean	LOD of labeled peptide [amol] 95% confidence interval
2,4-D-Peptide-Lys(biotin)-$CONH_2$	**(1)**	0.2 ± 0.1	1339.0 ± 865.1[b]	24547.1[b]	12882.5−46773.5
2,4-D-C6-Peptide-Lys(biotin)-$CONH_2$	**(3)**	0.7 ± 0.4[c]	201.4 ± 20.8	1698.2	1000.0−2951.2
2,4-D-C11-Peptide-Lys(biotin)-$CONH_2$	**(4)**	1.5 ± 0.3[d]	163.0 ± 44.6	380.2	239.9−602.6
2,4-D-PEG_{11}-Peptide-Lys(biotin)-$CONH_2$	**(12)**	0.2 ± 0.1	997.3 ± 589.9[e]	8709.6[f]	3162.3−23442.3
2,4-D-C6-PEG_{11}-Peptide-Lys(biotin)-$CONH_2$	**(13)**	0.4 ± 0.2	255.5 ± 91.9	1071.5	354.8−3235.9
2,4-D-C11-PEG_{11}-Peptide-Lys(biotin)-$CONH_2$	**(14)**	1.2 ± 0.4[d]	86.7 ± 27.9	169.8	49.0−588.8
Ac-HN-Lys(biotin)-Peptide-Lys(2,4-D)-$CONH_2$	**(19)**	0.4 ± 0.1	160.0 ± 36.8	676.1	144.5−3235.9
Ac-HN-Lys(biotin)-Peptide-Lys(C6−2,4-D)-$CONH_2$	**(20)**	0.6 ± 0.2	105.9 ± 28.5	269.2	74.1−977.2
Ac-HN-Lys(biotin)-Peptide-Lys(C11−2,4-D)-$CONH_2$	**(21)**	1.1 ± 0.3[d]	73.6 ± 17.5	107.2	24.0−467.7

[a] Peptides (sequence (N- to C-terminus): ASQLDYKMTDAGE) were double labeled with 2,4-D derivatives **(1)**, **(3)**, **(4)**, **(12)**, **(13)**, **(14)**, **(19)**, **(20)**, or **(21)** (see Figures S-1−S-4 in the Supporting Information) and lysine-*N*-ε-(biotin) (Lys(biotin)) during synthesis. The peptides were captured by anti-2,4-D antibody clone F6/C10 on polystyrene microplates and were detected via horseradish peroxidase-labeled streptavidin (N_{exp}: 6). Maximum optical density at 450 nm (MaxOD), steady-state dissociation constant K_D and limit of detection (LOD) were determined as described. Statistical analyses were performed by applying a one-way ANOVA and Bonferroni post hoc test. Results of other anti-2,4-D antibody clones tested were comparable. The best affinity of $K_D = 2.8 \times 10^{-11}$ M was obtained with antibody clone E2/G2 in combination with Ac-HN-Lys(Biotin)-Peptide-Lys(C11−2,4-D)-$CONH_2$. [b] Higher K_D and LOD than obtained with all other 2,4-D derivatives ($P < 0.05$) except with derivative **(4)** ($P > 0.05$). [c] Higher MaxOD than obtained with 2,4-D derivatives without spacer ($P < 0.05$). [d] Higher MaxOD than obtained with 2,4-D derivatives without and with C6-spacer ($P < 0.05$). [e] Higher LOD than obtained with all other 2,4-D derivatives ($P < 0.05$) except with derivatives **(1)** and **(2)** ($P > 0.05$). [f] Higher K_D than obtained with all other 2,4-D derivatives ($P < 0.05$) except with derivative **(1)** ($P > 0.05$).

and removed from proteinaceous substrates under mild conditions[18] (Figure 3C and Figure S-4 in the Supporting Information).

2,4-D dimedone **(18)** was reacted with the free amino terminus of cellulose-bound peptides after completion of the solid-phase

peptide synthesis. The labeled peptides were detected with biotinylated anti-2,4-D antibody and AlexaFluor680-labeled streptavidin. (Figure 3D). The 2,4-D tag was cleaved off with aqueous hydrazine which resulted in an almost complete removal of the 2,4-D tag after approximately 100 min. The integrity of the peptide was not compromised by the reversible labeling procedure as verified by subsequent reaction with peptide-specific antibodies.

Labeling of DNA with 2′-Deoxyuridine-5′-triphosphate (dUTP) Derivatives. The applicability of the 2,4-D tag was further expanded by establishing a DNA-labeling system based on dUTP derivatives of 2,4-D. 2,4-D-11-dUTP **(22)** (Figure 4A) was synthesized by reacting 5-(3-aminoallyl)-dUTP with the 2,4-D-C6-NHSS-ester **(5)** (see Figure S-4 in the Supporting Information) and incorporated into a DNA fragment via polymerase chain reaction. For comparison, biotin-11-dUTP and DIG-11-dUTP were used analogously. A ratio of 80:20 of 2,4-D-11-dUTP to dTTP was tolerated by Taq-DNA polymerase which was higher than for biotin-11-dUTP or DIG-11-dUTP (Figure 4B). In dot blot experiments on nylon membranes, approximately 9 amol of a 629 bp DNA fragment was detectable with the 2,4-D and the DIG systems, while the LOD for biotin-labeled DNA was 325 amol (Figure 4C).

DISCUSSION

We present a new indirect labeling system which is based on 2,4-dichlorophenoxyacetic acid (2,4-D) derivatives and 2,4-D-specific antibodies and which is suited for the specific labeling and sensitive detection of biomolecules in a variety of applications. In comparison with commonly used labeling reagents, the 2,4-D system excels in the achievement of particularly low limits of detection and, in general, compares favorably with the biotin/streptavidin and the DIG/anti-DIG systems. We attribute this high quality of performance to a consequent enhancement of the affinity between the 2,4-D tag and the monoclonal 2,4-D-specific antibodies which was achieved by linking aliphatic spacer moieties to the 2,4-D labeling molecules. This increase in affinity may be ascribed to a "spacer-binding" effect, which up to now has mainly been alleged as an undesirable interference in case of competition-type hapten immunoassays for the detection of low molecular weight analytes in environmental probes.[19,20] We decided to take advantage of this effect which is presumably due to the fact that antibodies induced by immunization with hapten−protein conjugates also recognize, to a certain extent, the linker structure between hapten and protein. Such linker structures are often made up of the carrier protein's lysine side chains. Yet, 2,4-D coupling to its target molecule merely via a lysine, as achieved by derivative **(19)**, did not result in the optimal binding partner for the 2,4-D-specific antibodies. The appendage of an additional aliphatic chain to the 2,4-D residue greatly improves the detectability by the antibodies when combining the spacered 2,4-D-labeling derivatives with the proper anti-2,4-D antibody. Not only peptides but also insulin, where the single lysine present is not reactive and the amino-termini are utilized in the labeling reaction,[21] and mucin

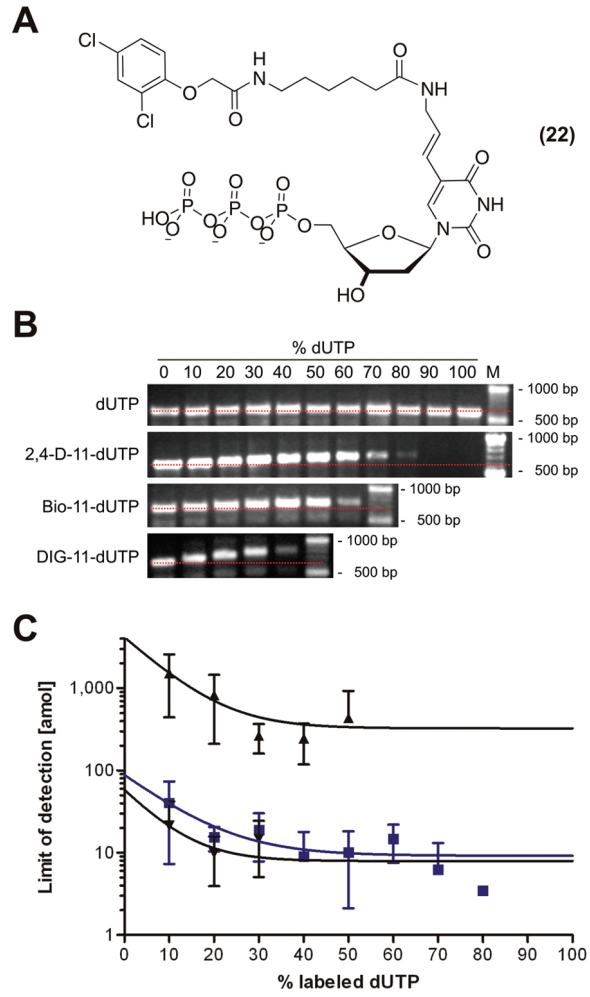

Figure 4. 2,4-D labeling of DNA. (A) A 2′-deoxyuridine-5′-triphosphate (dUTP) derivative equipped with a C6-spacered 2,4-dichlorophenoxyacetyl (2,4-D) moiety, 2,4-D-11-dUTP **(22)**, was prepared. (B) A 629 base pair (bp) DNA fragment of murine prion-DNA was amplified via polymerase chain reaction and Taq-DNA polymerase in the presence of different ratios of either unlabeled dUTP/dTTP (upper panel; 0−100% dUTP) or 2,4-D-, biotin-, or digoxigenin-(DIG)-11-dUTP/dTTP (0−100% labeled dUTP). The incorporation of the different dUTPs could be visualized by the band shifts of the PCR fragment toward higher molecular weights in the ethidium bromide stained agarose gel (dashed red line: level of unlabeled PCR fragment). (C) The PCR products labeled with **(22)**, biotin-11-dUTP, or DIG-11-dUTP were purified, and their concentration was determined after electrophoretic separation by densitometric scanning. The labeled DNA fragments were serially diluted and immobilized on nylon membrane. 2,4-D and DIG labels were detected by specific primary and AlexaFluor680-labeled secondary antibodies whereas biotin labels were visualized with streptavidin-AlexaFluor680 (N_{exp}: 2−4). Limits of detection (LOD; amol labeled DNA) (mean ± SD) were determined as described and plotted versus the respective dUTP/dTTP ratios used for amplification of the DNA fragments (■, **(22)**; ▼, DIG-11-dUTP; ▲, biotin-11-dUTP). The respective minimum LOD was determined by nonlinear regression analyses (one-phase exponential curve fit). The minimum LOD (lower plateau, 95% confidence interval) of 2,4-D-labeled DNA (9.2 amol, 2.7−15.7 amol) and DIG-labeled DNA (7.9 amol, −7.6 to +23.4 amol) was significantly lower than the minimum LOD of biotin-labeled DNA (324.5 amol, −21.4 to +670.4 amol) (one-way ANOVA, Bonferroni post hoc test, $P < 0.001$), whereas the minimum LOD of 2,4-D- and DIG-labeled DNA did not differ significantly ($P > 0.05$).

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labeled via the carbohydrate residues greatly benefit from the aliphatic spacers. In a situation, however, where the labeled target apparently mimics the situation of the hapten-carrier protein conjugate used for immunization very closely, the positive effect of the aliphatic spacers may be negligible. This is exemplified by our labeling of ovalbumin which has a blocked, acetylated N-terminus and can only be derivatized via its lysines.[22] The use of nonspacered 2,4-D **(2)** for labeling already establishes a C4-bridge between 2,4-D and the protein backbone. In this case, the sensitivity of detection cannot be improved by the use of derivatives **(5)** or **(6)** that comprise longer aliphatic spacers.

The 2,4-D system demonstrates its power best when used on substrate molecules that carry only one label, such as the synthetic peptides, or where numerous labels are rather distant from each other, as on mucin labeled via its carbohydrate residues. In connection with substrate molecules that can carry a high number of labels in relatively close proximity to each other, such as ovalbumin or the DNA fragment tested, the biotin and DIG/anti-DIG systems catch up to some extent. This may be due to space constraints on the labeled substrate molecule which allow binding of only a limited number of anti-2,4-D antibodies until the target molecule's surface is covered completely. By use of smaller detection reagents such as single-chain antibodies, the 2,4-D system can probably be pushed to even higher sensitivities.

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CONCLUSION

We have developed a new nonradioactive labeling system for the sensitive detection of biomolecules. The 2,4-D tag is highly versatile and robust and can be used with excellent results under all conditions generally involved in the labeling and detection of nucleic acids, peptides, proteins, or carbohydrates. The new labeling system is specifically suited for analytical applications which focus on small target molecules, such as peptides, and, as a novel member of the bioanalytical toolbox, greatly improves the possibilities for multilabel setups.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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