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Aminoethyl-substituted indole-3-acetic acids for the preparation of tagged and carrier-linked auxin

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Dedicated to Professor Iwao Ojima on the occasion of his 60th birthday

Abstract—Indole-3-acetic acid is an indispensable hormone (auxin) in plants and an important metabolite in humans, animals, and microorganisms. Here we introduce its 5- and 6-(2-aminoethyl)-derivatives for use in the design of novel research tools, such as immobilized and carrier-linked forms of indole-3-acetic acid and its conjugates with biochemical tags or biocompatible molecular probes. The aliphatic nitrogens of 5- and 6-(2-aminoethyl)indole were acetylated and the products were converted to the corresponding 3-(*N*,*N*-dimethylamino)methyl derivatives (gramines). These were reacted with cyanide. Saponification of the resulting acetonitriles was accompanied by *N*-deprotection to yield 5- and 6-(2-aminoethyl)indole-3-acetic acids. The latter were chemically stable and could be linked, via their amino groups, and without prior protection of their carboxyl moieties, to bovine serum albumin and to biotin, including appropriate spacer modules. One of the protein conjugates was used to elicit the formation of monoclonal antibodies, which were evaluated using the biotin conjugates in an enzyme-linked immunosorbent assay employing streptavidin-coupled alkaline phosphatase, and thus shown to recognize predominantly the indole-3-acetic acid moiety.

1. Introduction

Indole-3-acetic acid (IAA) is a universally distributed member of the class of phytohormones known as auxins the physiology of which has, during plant evolution, diversified into a pattern of unprecedented intricacy.[1] New approaches are needed to disentangle the multiple networks of metabolic and signaling pathways. Understanding these complexities is a prerequisite for targeted manipulation of crop development aimed at improving the quality and quantity of food and industrial supplies of plant origin. The complete picture would include the impact of IAA-forming plant-associated microorganisms of various systematic affiliations, including the nitrogen-fixing *Rhizobia* in the root nodules of legumes, and pathogens, such as tumor-causing *Pseudomonas* and *Agrobacterium* species.[2] Many microorganisms used in the fermentation industry form IAA as a metabolite with incompletely resolved biological functions.[3] This can be a nuisance when excessive quantities accumulate (for reasons currently under investigation) in some batches of beer and wine, because decomposition products arising during storage will spoil the beverage.[4] Microorganisms inhabiting the intestine are major sources of IAA in humans and mammals, though the compound is also produced by body tissues,[5] in particular in certain metabolic diseases, such as phenylketonuria.[6] IAA has no obvious vital functions in animals and is excreted via the urinary tract, in part after conjugation with glucuronic acid, taurine, or aliphatic amino acids.[7] Severe pathological effects will, however, ensue when detoxification and excretion fail, due to liver and/or kidney insufficiency. High IAA levels have, for instance, been reported to bind to serum albumin, interfering with its normal function as a transport protein for compounds, which cause toxic effects if circulating freely in the blood stream,[8] to induce proteins promoting tubulointerstitial kidney fibrosis,[9] and to attach to the 'aryl hydrocarbon receptor', a ligand-activated transcription factor which triggers the expression of genes involved in tumor promotion, immune suppression, endocrine

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disruption, wasting, and related syndromes resulting in morbidity and lethality.[10] The body's defense response is also weakened by damaging effects to neutrophilic leukocytes,[11] apparently because they contain peroxidase, which tends to convert IAA into at least one cytotoxic compound. Because of such toxic decomposition products,[12] IAA and its ring-substituted derivatives have also been suggested for cancer therapy, as prodrugs to be activated by in situ oxidation.[13] Some malignant cell lines already contain high levels of peroxidase,[14] to others horseradish peroxidase may be targeted by a variety of strategies.[13] Alternatively, IAA oxidation may be initiated by ionizing radiation[15] or by light in the presence of photosensitizers.[16,17] While this approach has shown very encouraging results in model systems,[15,16,18] its real-life application will require balancing therapeutic efficiency against undesired long-term toxicity. Routine monitoring of the pharmacodynamics of the therapeutic IAA derivative, including its binding to critical body proteins and, ideally, the construction of tagged derivatives preferentially absorbed by cancer cells could solve many of the problems.

The coupling of small molecules to such biochemical 'tags', to a variety of 'molecular probes' (e.g., biotin, fluorescent dyes, spin labels), to macromolecular carriers for eliciting antibody formation, or to sorbents for affinity purification of the immunoglobulins obtained (or of 'binding proteins' with physiological functions), requires the same general approach to be of practical use: an optimal set of structural elements identifying the basic molecule must remain unmodified and sterically accessible. In the case of IAA, research in this area has practically ended following attempts to produce specific antibodies.[19] This may, in part, be due to the disappointment with the commercial ELISA kit for IAA quantification, which was introduced in the mid 1980s (kit still available at http://www.agdia.com). The monoclonal antibodies used in this assay were found to be far too unspecific for IAA analysis in crude plant extracts.[20] Because of high cross reactivity with indoleaceturic acid, one of the main IAA metabolites in mammals,[6,7] the commercial assay is also not suitable for IAA analysis in medical and veterinary biochemistry. Moreover, cross-reactivity with the chemically unrelated α-linolenic acid, was demonstrated for a sister monoclonal[21] with otherwise similar binding properties,[22] and would clearly disqualify the kit for use in blood samples quite as well as in plant extracts.

The commercial antibodies were produced in response to IAA linked via its carboxyl group to a carrier protein. Functional groups engaged in the hapten–carrier bond are not exposed to the immune system when the conjugate is injected into an animal, and are thus not recognized by the antibodies formed.[23,24] To become recognizable, the carboxyl group must first be converted to a nonacidic derivative. In practice, the methyl ester is prepared using (highly toxic and carcinogenic) diazomethane. This derivatization is also required with other antibodies raised against carboxy-linked IAA, which have been produced in a number of laboratories.[22,25–27] If the hapten–carrier conjugate is created via the indole nitrogen,[27–29] the resulting antibodies do recognize free IAA. However, as simultaneous recognition of the NH group and the free carboxyl moiety, in a defined spatial relationship, is a logical prerequisite for distinguishing indole-3-acetic acid from its naturally occurring ester and amide conjugates,[30] and from a multitude of common aromatic and heterocyclic phytochemicals, cross-reactivity remains a problem with antibodies produced in response to both N-linked and carboxy-linked indole-3-acetic acid.[20,31] To circumvent these complications, 5-hydroxyindole-3-acetic acid, linked to a protein carrier via its phenolic OH-group, was used as an antigen.[32] This approach represents a significant improvement, but is not yet ideal because (1) coupling via the 5-OH group required temporary protection of the –COOH moiety, and its subsequent deprotection in the hapten–carrier conjugate could not be taken to completion; (2) the pyrrole NH-group in 5-hydroxyindole-3-acetic acid differs in acidity from the unsubstituted parent compound,[33] a feature likely to affect immunogenic properties; (3) 5-hydroxyindole-3-acetic acid is easily oxidized at physiological pH, resulting in contaminants, which have different immunogenic properties and could well be as toxic to experimental animals as the oxidation products of other 5-hydroxyindoles, such as 5-hydroxytryptophan[34] and 5-hydroxytryptamine.[35]

Here we introduce derivatives of indole-3-acetic acid that leave the carboxy and NH moieties of the IAA molecule intact and, as far as coupling to molecular probes, biochemical tags, and macromolecular carriers is concerned, share the useful properties of 5-hydroxyindole-3-acetic acid while overcoming its three main drawbacks—the need to temporarily protect the –COOH moiety during conjugation, the change in acidity of the pyrrole NH group,[36] and the sensitivity to oxidation. The derivatives are coupled to biotin and to a macromolecular carrier, which is used to elicit the formation of monoclonal antibodies, which are tested against the IAA–biotin tracer.

2. Results and discussion

2.1. Preparation of (2-aminoethyl)indole-3-acetic acids 5A and 5B

The procedures used (Fig. 1) followed a classical route for the preparation of ring-substituted indole-3-acetic acids.[37] Initial protection of the aliphatic nitrogen in aminoethylindoles **1A** and **1B** (each one prepared separately, as quoted in Section 4) was required. This was accomplished by introducing an *N*-acetyl group, which is easily removed in the last stages of the synthesis. Gradual addition of an equimolar amount of *N*-acetylimidazole, in benzene solution, afforded optimal yields of *N*-acetylated amines **2A** or **2B**, with no major concomitant acetylation noted at the indole nitrogen. During work-up, it was essential to avoid exposure of **2A** and **2B** to acid stronger than 4% H_3PO_4. For the subsequent conversion to gramines **3A** and **3B**, the use of the preformed Mannich reagent, *N*,*N*-dimethyl-*N*-methylene–ammonium chloride,[38,39] was preferred to the clas-

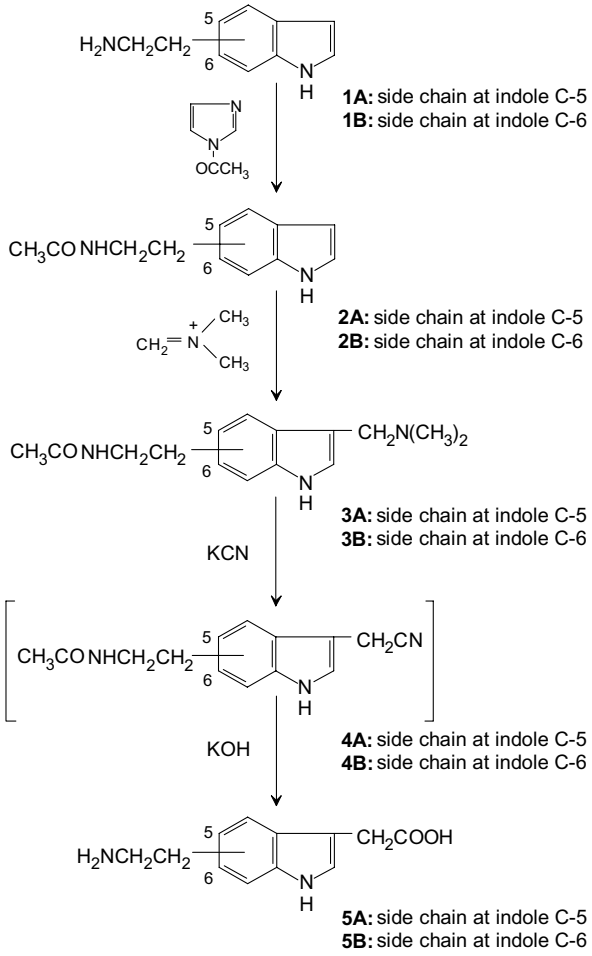

Figure 1. Synthesis of 5- and 6-(2-aminoethyl)indole-3-acetic acids. Either pure **1A** or pure **1B** (*not* a mixture of the two) were used as the starting compounds and there was no 5-to-6 (or vice versa) isomerization in the further sequence of the reactions. Compounds **4A** and **4B** were not isolated, but the occurrence of analogous acetonitrile intermediates has been unequivocally documented for a large number of closely related reactions.[37]

sical Mannich procedure. The following steps leading to aminoethylindole-3-acetic acids **5A** and **5B** were performed as a one-pot reaction. Acetonitriles **4A** and **4B**, implied to arise by reaction of **3A** and **3B** with excess KCN (in analogy with reactions discussed in Ref. 37), were not isolated because the simultaneously formed KOH initiated hydrolysis of the cyano group and deacetylation of the aliphatic nitrogen. The latter two reactions were taken to completion by adding excess KOH. The inorganic salts in the reaction mixtures were removed using a column of Sephadex LH-20 eluted with dilute aqueous ammonia. The resulting, water-soluble, ammonium salts of compounds **5A** and **5B** were converted to the sparingly soluble, free amino acids by adjusting their concentrated aqueous solutions to their isoelectric pH.

2.2. X-ray structure analysis of compounds 5A and 5B

X-ray structure analysis of positional isomers **5A** and **5B** confirms unambiguously that the molecules occur as

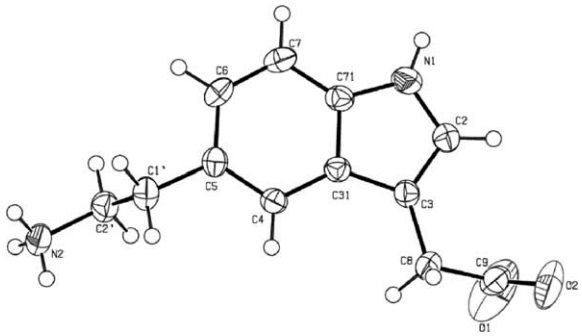

Figure 2. ORTEP view of the molecule of compound **5A** in its zwitterion form showing the crystallographic atom numbering scheme. Thermal ellipsoids are shown at 50% probability level.

zwitter ions (NH_3^+ and COO^- ions) in the solid state. The crystal structure of 5-isomer **5A** reveals one conformer, that of 6-isomer **5B** four different conformers in the same crystal. The overall conformation of the ammoniumethylindoleacetate ions studied is defined by torsion angles C2–C3–C8–C9 (for numbering see the ORTEP drawing of **5A** in Fig. 2) and C4–C5–C1′–C2′ (**5A**) or, respectively, C5–C6–C1′–C2′ (**5B**), which describe the orientation of the CH_2COO^- and ammoniumethyl chains with respect to the indole plane. In **5A** the CH_2COO^- bond is nearly coplanar to the indole ring (C2–C3–C8–C9 = −25.4°), in **5B** it is nearly perpendicular (C2–C3–C8–C9 ranging from −93.46° to −114.67°). The ammoniumethyl chain is tilted toward the indole plane in both **5A** and **5B**. Figure 3 shows the overlap of the four conformers of **5B** to illustrate the significantly different orientation of the ammoniumethyl chain in one of these conformers: in three conformers, the CH_2COO^- and ammoniumethyl chains are tilted to opposite sides of the indole ring whereas, in one conformer, these groups are turned to the same side of the indole plane (Fig. 3). Positional isomer **5A** crystallizes in the noncentrosymmetric space group $P2_1$ whereas isomer **5B** crystallizes in the centrosymmetric space group $P2_1/c$, but neither compound is chiral. Both compounds crystallize with water molecules; this results in a very complex two-dimensional network of hydrogen bonds involving the following donor and acceptor functionalities: N^+–H···O^-, N^+–H···O(water), O(water)–H···O^-. In the structure of isomer **5A** there

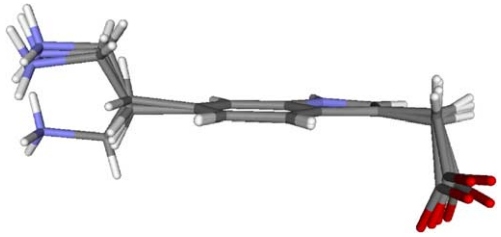

Figure 3. Overlap of the four conformations found in the asymmetric unit of compound **5B** (zwitterion form). In all conformations, the CH_2COO^- bond is positioned nearly perpendicular to the plane of the indole ring. The ammoniumethyl moieties are turned to the opposite side of the indole ring in three conformations, and to the same side in one conformation.

are significant intermolecular C–H···π and N–H···π interactions because of the almost perpendicular arrangement of neighboring indole rings (connected by translation along the crystallographic axis *a*) with the distances *d*(N–H···Cg(ring)) = 2.71 Å (angle = 138°) and *d*(C–H···Cg(ring)) = 2.67 Å (angle = 158°).

2.3. Coupling of 5- and 6-(2-aminoethyl)indole-3-acetic acid (5A and 5B) to mercaptosuccinylated bovine serum albumin

The methods are based on the use of bifunctional reagents, which contain (1) a succinimide ester group, which permits coupling to the aliphatic amino groups in **5A** and **5B**; (2) a maleimido moiety which permits coupling to free –SH groups in the carrier protein, and (3) an interspaced hydrocarbon residue. The following two representatives were tested: 4-(maleimidomethyl)-cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester and 4-(maleimido)butyric acid *N*-hydroxysuccinimide ester.

The course of the coupling reaction is outlined in Figure 4. The succinimide ester moiety in the bifunctional reagents was replaced by the amino group of compound **5A** or **5B**. In published hapten-conjugation procedures employing maleimide-substituted *N*-hydroxysuccinimide esters,[40] reaction times were kept short, and residues of the bifunctional reagents were removed by solvent extraction before adding the thiol component. This is not possible with condensation products **6A**,**B**, **7A**, and **7B**, which are extracted at least as readily as the bifunctional reagents. The latter were, however, completely consumed if only ca. 50% of their stoichiometric amount was employed, and if reaction times were extended (6 h for **6A** and **6B**, 1–3 h for **7A** and **7B**) beyond the limits, which are usually recommended. As indicated by TLC (solvent A_1), these prolonged reaction times did not lead to the accumulation of secondary products. In particular, within the time period noted above, there was no evidence for the instability of the maleimido groups in condensation products **6A**,**B**, **7A**, and **7B** in neutral solution, which appears to be a problem with maleimido-derivatized proteins.[41] Long-term stability, sufficient for isolation and physico-chemical characterization of compounds **6A**, **6B**, **7A**, and **7B** was, however, not expected due in part to the sensitivity of the maleimido moiety to nucleophilic addition and ring-opening solvolysis. They were thus immediately coupled to mercaptosuccinylated bovine serum albumin **9**.

The mercaptosuccinylation is necessary because native bovine serum albumin does not contain a sufficient number of thiol functions.[42] The derivatization was per-

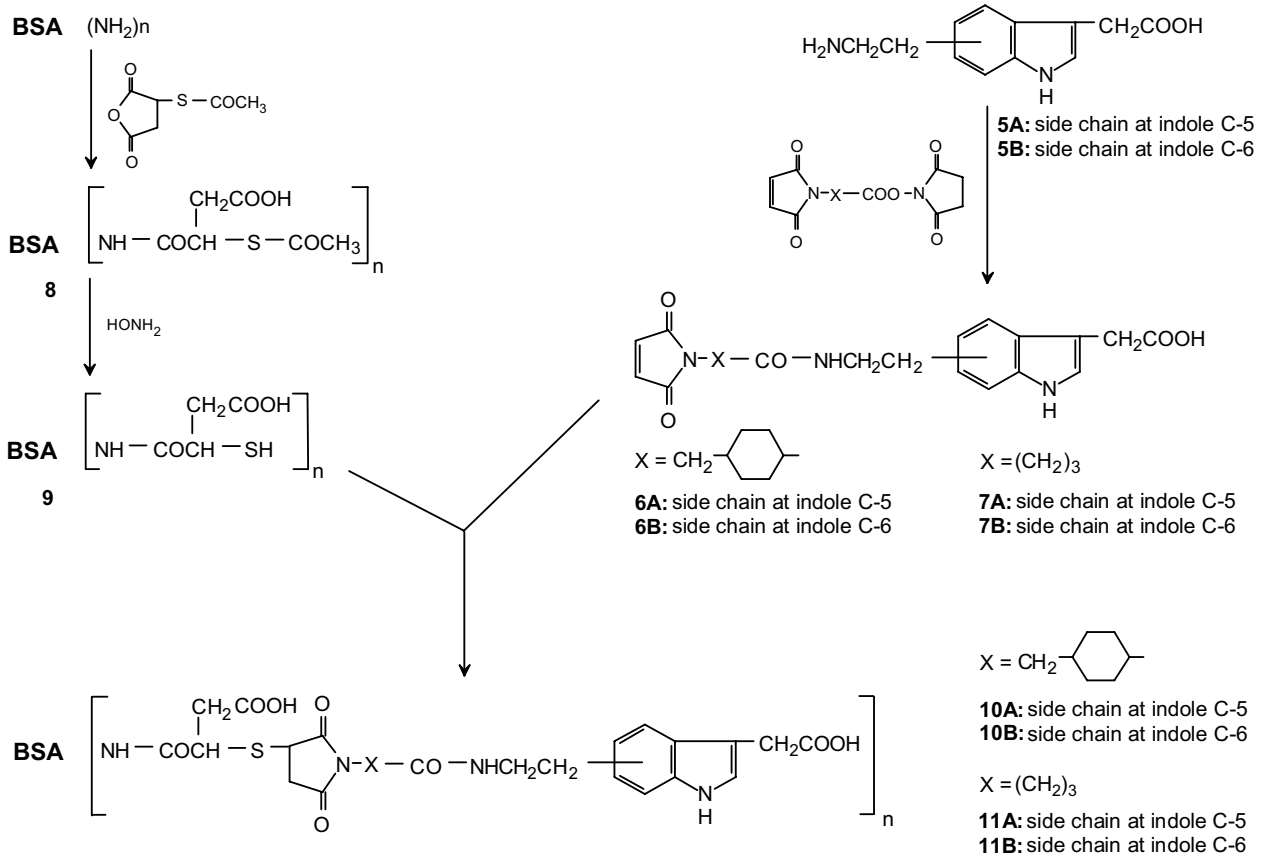

Figure 4. Coupling of 5- and 6-(2-aminoethyl)indole-3-acetic acids (**5A** and **5B**) to mercaptosuccinylated bovine serum albumin using bifunctional reagents composed of a maleimido and an *N*-hydroxysuccinimide ester moiety separated by an aliphatic or alicyclic spacer. Compounds **5A** and **5B** were independently synthesized (Fig. 1) and the original positions of the aminoethyl moiety at the indole nucleus remained unchanged during the further sequence of reactions. The mercaptosuccinyl moieties are attached to accessible ε-amino groups of lysine residues; their structure is presented as proposed by Klotz and Heiney.[43]

formed in two steps (Fig. 4). Condensation with *S*-acetylmercaptosuccinic anhydride[43] afforded *S*-acetylmercaptosuccinylated bovine serum albumin (**8**), which could be stored for extended periods of time. Immediately before use, the protective *S*-acetyl groups were removed to yield **9**, employing hydroxylamine as a catalyst.[43] In most cases, the crude reaction mixture was used for condensation with **6A**,**B**, **7A**, and **7B**. In that case, the hydroxylamine and the thiol groups in **9** compete for nucleophilic attack at the maleimido moieties. The condensation with the hydroxylamine could, however, be suppressed by diluting the reaction mixture before it was added to **6A**,**B**, **7A**, or **7B**. Mercaptosuccinylated protein **9** could also be purified by gel filtration (data not shown). This efficiently removed the hydroxylamine, but was time-consuming and, in the present context, showed no practical advantages.

As shown using the Ellman assay,[44] mercaptosuccinylated protein **9** was completely consumed in the condensation with **6A**,**B**, **7A**, or **7B**. S–S linked dimers and/or oligomers (formed by oxidative processes) of protein **9** were, however, expected as by-products, along with residues of low molecular weight reagents. The crude protein conjugates **10A**,**B**, **11A**, and **11B** were thus purified by gel filtration using Sephadex G-50. Elution with a pH 7 buffer containing 6 M urea proved to be essential for efficient separation. To remove buffer salts and urea, the respective fractions were filtered through a molecular-size-selective membrane to a small residual volume, followed by a sequence of dilution and re-filtration steps. In the initial experiments, redistilled water was used in the dilution step. However, the completely desalted protein conjugate precipitated in some cases. In most experiments, the water was thus replaced by 20 mM Na–phosphate buffer, pH 7.0. The final solution obtained was lyophilized. The phosphate content (including water of crystallization) was determined by lyophilizing an equal volume of buffer.

The average number of hapten moieties (11–16) in the protein conjugates **10A**,**B**, **11A**, and **11B**, as estimated spectrophotometrically, varied somewhat from batch to batch, but remained within the range recommended for antigens (8–25).[23] The degree of hapten substitution was also monitored by MALDI-TOF mass spectrometry. The mass profile of conjugate **10B** is shown in Figure 5, as an example. The ion intensity maximum corresponds to a conjugate containing [(73,501 − 66,667)/569] = 12 hapten units, in good agreement with the result obtained by spectrophotometric analysis. While the mass spectrum confirmed the absence of unreacted protein **9**, the broad and somewhat trailing mass profile suggests, as expected, that a range of conjugates are represented containing a variable number of hapten moieties. Conjugate **10B** was used for the production of monoclonal antibodies in mice.

2.4. Biotin conjugates 13A and 13B of 6-(2-aminoethyl)-indole-3-acetic acid (5B)

To permit the evaluation of the antibodies obtained, a labeled tracer was required. We used biotin as a label

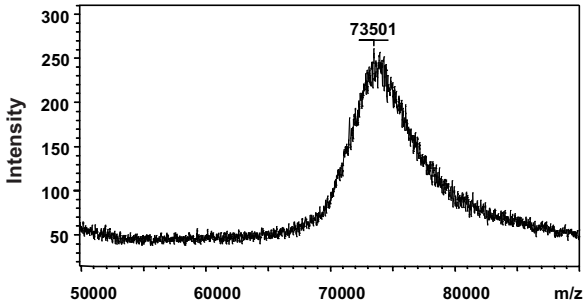

Figure 5. Mass spectrum of protein conjugate **10B** using a MALDI-TOF mass spectrometer confirming the change in molecular mass of bovine serum albumin as a result of the addition of approximately 12 aminoethylindole-3-acetic acid groups linked via a spacer (calculated molecular mass per hapten unit: 569). Sinapinic acid was used as the matrix, and bovine serum albumin was used for calibration. The spectrum represents the sum of consecutive laser shots (smoothed over 20 data points).

which can be detected with high sensitivity using streptavidin-linked alkaline phosphatase and *p*-nitrophenylphosphate as a chromogenic substrate. The two tracers tested here were prepared by condensation of amino acid **5B** with a slight excess of succinimide esters **12A** or **12B**, which contain biotin and one or two 6-aminohexanoyl spacer modules (Fig. 6). The by-product, *N*-hydroxysuccinimide, was extracted with ethyl acetate from the evaporated reaction mixture. The residue was passed through a C_{18}-reversed phase cartridge to yield biotin conjugates **13A** and **13B** in sufficient purity for use as tracers.

2.5. Immunoassays

Among the mouse hybridoma lines so far screened (details not shown), 24 produced monoclonal antibodies against the original antigen **10B**. For quantitative evaluation, the immunoglobulins were immobilized to the wells of microtiter plates and exposed to tracer **13B**. Using detection with streptavidin-linked alkaline phosphatase and *p*-nitrophenylphosphate (see Section 4), the amount of tracer bound by the antibody translated into an increase in the absorbance at 405 nm from the release of free *p*-nitrophenol. Individual antibodies were screened using a 1:10 dilution of the antibody supernatant and a fixed concentration (0.5 ng/μL) of **13B**. Twelve of the original monoclonals afforded a final absorbance (at 405 nm in the standard set-up) larger than 0.40, while the absorbance values for the other 12 lines ranged from 0.18 to 0.39. The latter were classified as very-low-affinity antibodies and not further investigated. The other 12 monoclonals (at a 1:30 dilution), were exposed to a range of concentrations of tracer **13B**. Three typical examples for the dose–response curves obtained are shown in Figure 7a. The performance of the elite four (33.3%) monoclonals closely corresponded to the upper curve (high-affinity antibodies); the medium curve was representative for five (41.7%), and the lower curve for three (25%) antibody lines. For all 12 monoclonals, exposure to at least 10^1 nmol/L of **13B** resulted in sufficient binding to the

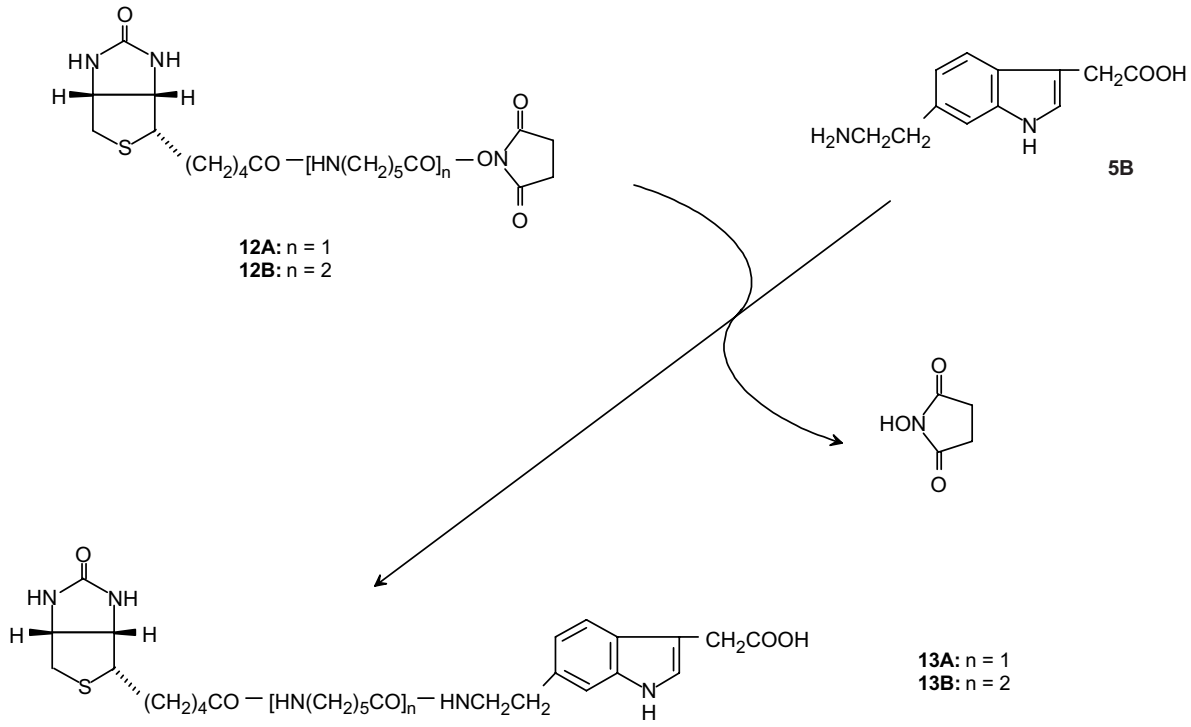

Figure 6. Coupling of biotin to 6-(2-aminoethyl)indole-3-acetic acid via a spacer composed of one or two 6-aminohexanoyl moieties.

immunoglobulin to produce a measurable increase in absorbance. The response at higher concentrations varied within wide limits and, for the high-affinity antibodies, approached saturation around 10^3 nmol/L. More detailed experiments with the high-affinity antibodies established a detection limit of 50 pg for **13B**, which corresponds to the sensitivity routinely exhibited with bench-top mass spectrometers.[45] The extent that the design of the tracer influenced the sensitivity of the assay is shown in Figure 7b. Assayed against the same high-performing monoclonal, a drastically reduced response was observed when **13B**, with a $C_{12}N_2$-spacer separating the biotinoyl and 6-(2-aminoethyl)indole-3-acetic acid moieties, was replaced by **13A** with a shorter C_6N-spacer. In analogy with published results on comparable systems,[46] the optimal length of the spacer module may be expected to depend on (1) the topology of the active site of the monoclonal antibody which binds to the 6-(2-aminoethyl)indole-3-acetic acid moiety in **13A** or **13B**, (2) the topology of the streptavidin site,[47] which binds their biotin moieties, and (3) the sterical and physico-chemical complementarity of the contact surfaces of the two proteins interacting with the biotin conjugates. As the influence of factors (1) and (3) could not be predicted, a suitable length for the spacer moiety had to be determined experimentally.

Monoclonal antibodies often bind to small sections of the surface of the antigen. Some clones might thus be directed against the residues of the bifunctional reagent by which the 6-(2-aminoethyl)indole-3-acetic acid moieties in **10B** were linked to the protein carrier. This is, however, unlikely for high-affinity antibodies exemplified by the upper curve in Figure 7a because (1) immunoglobulins recognizing parts of a cyclohexane residue would be expected to bind with low affinity, if at all, to the aliphatic spacer in **13B** and (2) antibodies attached to the spacer would reduce its effective length and thus jeopardize binding of the biotin moiety to streptavidin. These arguments strongly suggest that at least our high-affinity monoclonal antibodies are directed against 6-(2-aminoethyl)indole-3-acetic acid. The specific parts of the molecule, which are recognized by the individual clones, will be the subject of future detailed studies.

3. Conclusion

In contrast to many other ring-substituents, attaching a 2-aminoethyl group at positions 5 or 6 did not introduce obvious instability into the IAA molecule. The derivatives obtained (**5A** and **5B**) were easy to store and to handle and could be coupled, via their amino groups and without protection of the carboxyl moiety, to biotin and bovine serum albumin under conditions sufficiently mild for use with sensitive biomolecules. The crystal structures of compounds **5A** and **5B**, as well as the fact that antibodies formed in response to protein conjugate **10B** recognized biotin conjugates **13A** and **13B** demonstrates that the 2-aminoethyl moiety (1) does not 'eclipse' the essential structural elements of the IAA moiety including the carboxyl group and (2) together with the hydrocarbon spacers employed prefers extended conformations (i.e., no back-folding over the IAA moiety) keeping the IAA moiety exposed as a target for molecular recognition. Using essentially the same uncomplicated protocols as outlined here, it should be possible to couple aminoethyl–IAAs **5A** and **5B** to a variety of tags and molecular probes used in biochemical

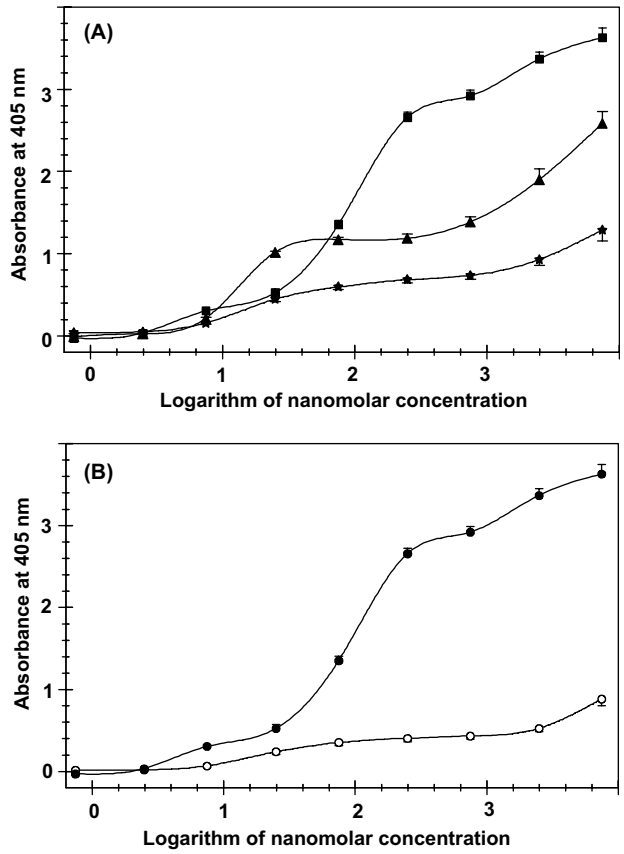

Figure 7. Performance of selected monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA). The antibodies, raised against protein conjugate **10B** of 6-(2-aminoethyl)indole-3-acetic acid, were immobilized at the walls of microtiter plates and exposed to a range of concentrations (abscissa) of biotinylated 6-(2-aminoethyl)indole-3-acetic acid. The bound biotin moiety was then capped with streptavidin–alkaline phosphatase and the enzyme immobilized in this way was detected by exposure to *p*-nitrophenyl phosphate. The absorbance at 405 nm (ordinate) reflects the amount of *p*-nitrophenol liberated under standard conditions (refer to Section 4 for further details) and is a measure for the amount of biotinylated 6-(2-aminoethyl)indole-3-acetic acid captured by the antibody. The data points represent the arithmetic means of four replicate measurements; the error bars indicate the standard error of the mean. Panel A shows the affinity of selected monoclonal antibodies with high (squares), medium (triangles) and low (stars) affinity toward biotin conjugate **13B**. In panel B we compare the performance of the high-affinity monoclonal antibody shown in panel A (upper curve) with biotinylated 6-(2-aminoethyl)indole-3-acetic acids containing spacer modules of different lengths. Open circles: conjugate **13A** (C_6N-spacer). Filled-in circles: conjugate **13B** ($C_{12}N_2$-spacer).

and biomedical research. The expectation, in the 1980s, that immunoassays would permit fast and reliable quantification of IAA in any crude extract of biological material appears to be unrealistic even with antibodies elicited by haptens **5A** and **5B**, but more targeted applications remain promising and should be explored.

4. Experimental

Melting points were determined in open capillaries and were not corrected. NMR spectra were recorded (at 20 °C unless stated otherwise) on a Varian (Palo Alto, CA, USA) Gemini 300 spectrometer operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C. Chemical shifts are reported in parts per million downfield from tetramethylsilane. Assignments are based on the analysis of coupling patterns, dissociation-induced shifts (for **5A** and **5B**), and ^{1}H–^{13}C shift correlation spectra. High resolution, positive ion mass spectra were recorded on an Applied Biosystems (Foster City, CA, USA) QStar quadrupole time-of-flight instrument, with electrospray ionization, using the $[M+H]^+$ ions of caffeine ($C_8H_{11}N_4O_2$; m/z = 195.0882) and reserpine ($C_{33}H_{41}N_2O_9$; m/z = 609.2812) and the $[M+3H]^{3+}$ ion of porcine angiotensinogen fragment 1–14 (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH+3H; $C_{85}H_{126}N_{21}O_{20}$; m/z = 586.9830) as internal standards. MALDI-TOF mass spectra of hapten–carrier conjugates were obtained on a Bruker Daltonics Biflex III instrument equipped with an N_2 laser, using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix, and bovine serum albumin for calibration.

Column chromatography on silica gel 60 (E. Merck, Darmstadt, Germany; particle size 0.063–0.2 mm) and on Sephadex LH-20 was performed at room temperature. Chromatography and further work-up of proteins and protein conjugates was carried out at +4 °C. Reversed phase HPLC was on a column (25 cm × 0.46 mm i.d.) of C_{18}-coated silica gel (particle size 5 μm), operated at a temperature of 22 °C, at a flow rate of 1 mL/min, using the following solvents: 0.1 M aqueous HOAc/MeOH (100:5; solvent I), H_2O/MeOH (100:5; solvent II), and MeOH (solvent III). Elution of compounds **3A**,**B**, **5A**, and **5B** began with solvent I (2 min), followed by a linear gradient of solvent III to a level of 50% at 30 min; this concentration was maintained for 8 min. Elution of compounds **2A** and **2B** was initiated with 20% solvent III in solvent II (2 min). The concentration of solvent III was then raised to 50% (linear gradient; 30 min) and maintained for 8 min. The UV absorbance of the effluent was monitored at 273 nm using a diode array detector (Wellchrom K-2800, Dr. ing. Herbert Knauer GmbH, Berlin, Germany). TLC was on silica gel GF_{254} (E. Merck) developing with CH_2Cl_2/MeOH/HOAc (90:10:1, solvent A_1; 70:30:5, solvent A_2), 2-PrOH/EtOAc/25% NH_3 (aq) (35:45:20, solvent B), CH_2Cl_2/MeOH/Et_3N (90:8:2, solvent C), or CH_2Cl_2/MeOH (95:5, solvent D). In addition to detection by UV absorption, the following spray reagents were used: the Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in a 1:1 mixture of EtOH and 35% HCl) for indolic compounds, 10% H_2SO_4 in EtOH (with heating) for substituted gramines, and *p*-dimethylaminocinnamaldehyde (0.2% in EtOH mixed, immediately before use, with an equal volume of 2% H_2SO_4 in EtOH) for the biotin moiety.[48]

Commercial, analytical grade chemicals and reagents were used except for 5-(2-aminoethyl)indole (**1A**) and 6-(2-aminoethyl)indole (**1B**), which were prepared according to Troxler et al.[49] The Mannich reagent, *N*,*N*-dimethyl-*N*-methylene–ammonium chloride was prepared from freshly redistilled acetyl chloride and

commercial *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane,[38] dried in vacuo, and used immediately. Of the solvents used, Et_2O and tetrahydrofuran (THF), must be free of peroxides.

All reactions involving proteins with free thiol groups were performed under nitrogen, using buffers and reagent solutions, which were previously saturated with N_2 or degassed with He. NaOH (0.5 M) was used for pH-adjustments. The reagent referred to as 'hydroxylamine stock' contained 500 mM $NH_2OH{\cdot}HCl$, 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM tris(hydroxymethyl)aminomethane, and HCl to pH 7.00. Protein concentrations were estimated using the Bradford reagent.[50] Free thiol groups were determined with Ellman's reagent [39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 10 mL 0.1 M phosphate buffer, pH 7.0].[44] The number (*n*) of 5- or 6-(2-aminoethyl)indole-3-acetic acid residues per molecule of protein conjugate was estimated from its UV absorbance using Eq. 1:

$$n = \frac{A\mathrm{MW_p} - \varepsilon_p c^* d}{\varepsilon_h c^* d - A\mathrm{MW_h}} \quad (1)$$

wherein A = absorbance at 279 nm; c^* = concentration of the conjugate in g L^{-1}; MW_p = molecular weight of the protein moiety (67,000); MW_h = molecular weight of the hapten including spacer and 2-mercaptosuccinyl moieties (569 for **10A** and **10B** and 515 for **11A** and **11B**); ε_p = molar absorptivity of the protein moiety at 279 nm (4.472×10^4); ε_h = molar absorptivity of the hapten at 279 nm (i.e., of 5- or 6-(2-aminoethyl)indole-3-acetic acid as the spacer and 2-mercaptosuccinyl moieties have negligible absorptivity at that wavelength); d = length of the optical path in cm.

Enzyme-linked immunosorbent assays (ELISA) were performed using Immuno 96-MicroWell Maxisorb plates (Nunc, Roskilde, Denmark) and the following buffered salt solutions: TBS [tris-buffered saline: 50 mM NaCl, 2 mM tris-(hydroxymethyl)aminomethane, HCl to pH 7.2], TTBS (0.1% Tween-20 in TBS), PBS (phosphate-buffered saline: 2.7 mM KCl, 147 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4).

4.1. Organic syntheses (Fig. 1)

4.1.1. 5-(2-Acetamidoethyl)indole (2A). A suspension of **1A** (1.41 g, 8.81 mmol) in dry benzene (43 mL) was stirred at 55 °C until most of the amine dissolved. The heating was then removed and a solution of *N*-acetylimidazole (971 mg, 8.82 mmol) in dry benzene (38 mL) was added dropwise, through 1 h. After further 30 min of stirring, EtOAc was added to dissolve the product, and the mixture was partitioned against 4% H_3PO_4 (4×50 mL), 5% $NaHCO_3$ (2×25 mL), and brine (2×15 mL), in this order. The organic phase was dried (anhydrous Na_2SO_4) and evaporated to yield 1.55 g (87%) of the title compound as a yellowish solid (no sharp mp), which remained amorphous on further purification by column chromatography (solvent D). Purity by HPLC (t_R = 21.5 min) 99.6%. ^{1}H NMR [$(CD_3)_2CO$]: δ 10.30 (1H, br s, indole NH), 6.97 (1H, dd, $J_{6,7}$ = 8.3 Hz, $J_{4,6}$ = 1.3 Hz, H-6), 6.40 (1H, m, H-3), 7.28 (1H, t, $J_{1,2} = J_{2,3}$ = 2.7 Hz, H-2), 7.33 (1H, d, H-7), 7.38 (1H, s, H-4), 2.83 (2H, t, J = 7.3 Hz, Ar–CH_2), 3.43 (2H, td, $J_{CH_2,NH}$ = 6.0 Hz, CH_2N), 7.23 (1H, br s, side chain NH), 1.87 (3H, s, CH_3). ^{13}C NMR (CD_3OD): δ 125.9 (C-2), 102.2 (C-3), 123.4, 121.0 (C-4,6), 130.8 (C-5), 112.2 (C-7), 129.8 (C-3a), 136.6 (C-7a), 36.8 ($ArCH_2$), 43.1 (CH_2N), 173.4 (C=O), 22.7 (CH_3). HRMS *m*/*z* 203.1176 ($[MH]^+$, $C_{12}H_{15}N_2O$ requires 203.1184), 144.0799 ($[MH-CH_3CONH_2]^+$, $C_{10}H_{10}N$ requires 144.0813).

4.1.2. 6-(2-Acetamidoethyl)indole (2B). Compound **1B** (1.32 g, 8.25 mmol) was reacted with an equimolar amount of *N*-acetylimidazole as described for **2A** to yield the title compound (1.37 g, 82%) as a yellow solid. Column chromatography on silica gel (solvent D) afforded colorless crystals, mp 74–75 °C, purity by HPLC (t_R = 25.4 min) 99.7%. ^{1}H NMR [$(CD_3)_2CO$]: δ 10.39 (1H, br s, indole NH), 7.26 (1H, t, $J_{1,2} \sim J_{2,3}$ = 2.7 Hz, H-2), 6.43 (1H, m, H-3), 7.48 (1H, d, $J_{4,5}$ = 8 Hz, H-4), 6.90 (1 H, d, H-5), 7.28 (1H, s, H-7), 7.38 (1H, br s, *NH*CH_2), 2.86 (2H, t, J_{vic} = 7.5 Hz, Ar–*CH*$_2$), 3.46 (2H, td, $J_{CH_2,NH}$ = 6.3 Hz, side chain NH), 1.90 (3H, s, CH_3CO). ^{13}C NMR (CD_3OD): δ 125.6 (C-2), 102.4 (C-3), 121.4, 121.5 (C-4,5), 133.5 (C-6), 112.2 (C-7), 128.2 (C-3a), 138.1 (C-7a), 37.0 ($ArCH_2$), 42.9 (CH_2N), 173.3 (C=O), 22.8 (CH_3). HRMS *m*/*z* 203.1186 ($[MH]^+$, $C_{12}H_{15}N_2O$ requires 203.1184), 144.0719 ($[MH-CH_3CONH_2]^+$, $C_{10}H_{10}N$ requires 144.0813).

4.1.3. 5-(2-Acetamidoethyl)-*N*,*N*-dimethyl-indole-3-methanamine [5-(2-acetamidoethyl)gramine; 3A]. The Mannich reagent, *N*,*N*-dimethyl-*N*-methylene–ammonium chloride, prepared from acetyl chloride (1.60 mL, 22.50 mmol) and *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (3.07 mL, 22.50 mmol), was suspended in dry CH_2Cl_2 (15 mL) and a solution of **2A** (3.79 g, 18.74 mmol) in the same solvent (15 mL) was added. After 20 min of stirring at room temperature, with exclusion of moisture, H_2O (15 mL) was added to decompose excess Mannich reagent. The reaction mixture was transferred to a separatory funnel using, in sequence, EtOAc (ca. 150 mL) and H_2O (ca. 30 mL) to dissolve oily precipitates. The aqueous phase was made strongly alkaline (35% aq NaOH) and the organic phase was collected. Maintaining a strongly alkaline aqueous phase, the remaining title compound was extracted with EtOAc (4×100 mL). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue (5.0 g) was passed through a column of silica gel (400 g) eluted with solvent C, to yield the title compound (4.15 g, 85%) as white crystals. Recrystallization from acetone/cyclohexane raised the mp to 138–139 °C; purity by HPLC (t_R = 15.3 min) 97.5%. ^{1}H NMR (CD_3OD): δ 7.55 (1H, s, H-4), 7.38 (1H, d, $J_{6,7}$ = 8.3 Hz, H-7), 7.26 (1H, s, H-2), 7.08 (1H, d, H-6), 2.96 (2H, t, J = 7.3 Hz, Ar–5-CH_2), 3.52 (2H, t, CH_2NHAc), 1.99 (3H, s, acetyl CH_3), 3.74 (2H, s, Ar–3-CH_2), 2.35 (6H, s, $2 \times NCH_3$). ^{13}C NMR (CD_3OD): δ 126.6 (C-2), 111.4 (C-3), 129.6 (C-3a), 119.5 (C-4), 130.9 (C-5), 123.7 (C-6), 112.4 (C-7), 137.0 (C-7a), 36.9 (Ar–5-CH_2), 43.0 (CH_2NAc), 173.3 (CO), 22.7 (acetyl CH_3), 54.8 (Ar–3-CH_2), 45.1 ($2 \times NCH_3$). HRMS *m*/*z*

260.1779 ([MH]$^+$, $C_{15}H_{22}N_3O$ requires 260.1763), 215.1160 ([MH−HN$(CH_3)_2$]$^+$, $C_{13}H_{15}N_2O$ requires 215.1184), 173.1110 ([MH−HN$(CH_3)_2$−CH_2CO]$^+$, $C_{11}H_{13}N_2$ requires 173.1079), 156.0858 ([MH−HN-$(CH_3)_2$−CH_3CONH_2]$^+$, $C_{11}H_{10}N$ requires 156.0813).

4.1.4. 6-(2-Acetamidoethyl)-*N*,*N*-dimethyl-indole-3-methanamine [6-(2-acetamidoethyl)gramine; 3B]. *N*,*N*-Dimethyl-*N*-methylene–ammonium chloride prepared from acetyl chloride (2.71 mL, 38.07 mmol) and *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (5.19 mL, 38.07 mmol) was reacted with **2B** (7.0 g, 34.61 mmol) as described for the preparation of **3A**. Chromatography of the crude product (7.5 g) on a column of silica gel (500 g, solvent C) afforded the title compound (6.74 g, 75%) as white crystals. Recrystallization from acetone/cyclohexane raised the mp to 102–105 °C (sintering at 100–102 °C); purity by HPLC (t_R = 14.8 min) 99.0%. ^{1}H NMR (CD_3OD): δ 7.53 (1H, d, $J_{4,5}$ = 8.1 Hz, H-4), 7.20 (1H, s, H-7), 7.14 (1H, s, H-2), 6.92 (1H, dd, $J_{5,7}$ = 1.2 Hz, H-5), 2.85 (2H, t, J = 7.3 Hz, Ar–6-CH_2), 3.41 (2H, t, CH_2NHAc), 1.90 (3H, s, CH_3CO), 3.63 (2H, s, Ar–3-CH_2), 2.42 (6H, s, N$(CH_3)_2$). ^{13}C NMR (CD_3OD): δ 138.5 (C-7a), 133.9 (C-6), 128.2 (C-3a), 126.2 (C-2), 121.6, 119.9 (C-4, C-5), 112.4 (C-7), 111.7 (C-3), 37.1 (Ar–6-CH_2). 43.0 (CH_2NHAc), 54.9 (Ar–3-CH_2), 45.2 [N$(CH_3)_2$], 173.5 (CO), 22.8 (acetyl CH_3). HRMS *m*/*z* 260.1789 ([MH]$^+$, $C_{15}H_{22}N_3O$ requires 260.1763), 215.1146 ([MH−HN$(CH_3)_2$]$^+$, $C_{13}H_{15}N_2O$ requires 215.1184), 173.1130 ([MH−HN-$(CH_3)_2$−CH_2CO]$^+$, $C_{11}H_{13}N_2$ requires 173.1079), 156.0864 ([MH−HN$(CH_3)_2$− CH_3CONH_2]$^+$, $C_{11}H_{10}N$ requires 156.0813).

4.1.5. 5-(2-Aminoethyl)indole-3-acetic acid (5A). The following synthesis was performed in a fume hood, as toxic HCN gas is formed as a by-product. To a solution of **3A** (3 g, 11.57 mmol) in 95% EtOH (43 mL) and H_2O (10.7 mL), KCN (7.5 g, 115.7 mmol) was added and the mixture was refluxed until TLC (solvent B) indicated complete conversion of **3A** to nitrile **4A** and compounds formed by its hydrolysis (ca. 48 h). To complete the saponification, KOH pellets (18 g) and H_2O (6.5 mL) were added to the cooled reaction mixture and boiling was resumed for 14 h. The mixture was diluted with water (60 mL) and colored by-products were extracted with diethyl ether (3 × 65 mL). The organic phase was back-extracted with 0.1 M NaOH (2 × 20 mL), which was added to the aqueous phase of the previous partition. The pH of the combined aqueous phases was adjusted to 10.5 using concentrated HCl. The white, inorganic, precipitate (likely SiO_2 from silicates extracted from glassware by the strongly alkaline reaction mixture) formed was removed by centrifugation at +3 °C. The supernatant was concentrated to two-thirds its original volume and, in three equal aliquots, passed through a column (57 × 2.5 cm i.d.) of Sephadex LH-20 packed in, and eluted with, dilute NH_3 (1 part of the commercial 25% NH_3 solution in 100 parts of H_2O). Most of the inorganic salts from the reaction mixture eluted before the title compound (elution volume 250–350 mL); overlapping fractions were pooled and rechromatographed. Evaporation of the effluent afforded the ammonium salt of **5A** (2.71 g) as an amorphous, extremely hygroscopic, foam. This was dissolved in dilute ammonia (same as the eluent for the Sephadex column; 10 mL) and the pH was adjusted to 8 (3 N HCl), when the amino acid precipitated in its zwitterionic form. The latter was collected by centrifugation (1.78 g). Partial evaporation of the supernatant afforded a second crop of **5A** (0.31 g). Overall yield: 2.09 g (83%). Repeated crystallization from H_2O (ca. 13 mL/g) afforded off-white leaflets, mp 236–237 °C (with decomposition). Purity by HPLC (t_R = 13.5 min): 99.3% pK_1 = 6.4; pK_2 = 9.4. ^{1}H NMR (CD_3OD/D_2O, 1:1, 50 °C): δ 7.28 (1H, s, H-2), 7.58 (1H, s, H-4), 7.13 (1H, d, $J_{6,7}$ = 8.3 Hz, H-6), 7.49 (1H, d, H-7), 3.69 (2H, s, CH_2COOH), 3.33 (2H, t, J = 7.3 Hz, CH_2N), 3.12 (2H, t, ArCH_2). ^{13}C NMR (CD_3OD/D_2O, 1:1, 50 °C): δ 125.5 (C-2), 112.0 (C-3), 129.2 (C-3a), 120.1 (C-4), 127.8 (C-5), 123.4 (C-6), 113.1 (C-7), 136.9 (C-7a), 34.4 (Ar–5-CH_2), 42.6 (CH_2N), 35.6 (Ar–3-CH_2), 182.0 (COOH). UV (EtOH/H_2O, 1:1) λ (log ε) 226.5 (4.41; maximum), 278 (3.63; shoulder), 279 (3.62; used for quantification of bound **5A** in **10A** and **11A**), 285 (3.65; maximum), 291 (3.59; shoulder). HRMS *m*/*z* 219.1180 ([MH]$^+$, $C_{12}H_{15}N_2O_2$ requires 219.1134), 202.0900 ([MH−NH_3]$^+$, $C_{12}H_{12}NO_2$ requires 202.0868), 156.0875 ([MH−NH_3−HCOOH]$^+$, $C_{11}H_{10}N$ requires 156.0813).

4.1.6. 6-(2-Aminoethyl)indole-3-acetic acid (5B). In analogy to the preparation of compound **5A**, a solution of gramine **3B** (1.8 g, 6.94 mmol) and KCN (4.52 g, 69.40 mmol) in a mixture of 95% ethanol (25.8 mL) and water (6.4 mL) was refluxed for 48 h and hydrolysis of the intermediate **4B** was completed by adding KOH (10.8 g) and H_2O (3.9 mL) and boiling (14 h). Further work-up as above afforded the ammonium salt of the title compound (1.89 g), which was converted to the free amino acid (1.30 g, 86%). Repeated crystallization from H_2O (ca. 25 mL/g) afforded off-white leaflets, mp 262–263 °C (with decomposition). Purity by HPLC (t_R = 14.8 min): 99.3%. pK_1 = 6.2; pK_2 = 9.3. ^{1}H NMR (CD_3OD/D_2O, 1/1, 50 °C): δ 7.67 (1H, d, $J_{4,5}$ = 8.0 Hz, H-4), 7.04 (2H, d, H-5), 7.37 (1H, s, H-7), 7.26 (1H, s, H-2), 3.68 (2 H, s, CH_2COOH), 3.28 (2H, t, J = 7.2 Hz, CH_2N), 3.10 (2H, t, Ar–CH_2). ^{13}C NMR (CD_3OD/D_2O, 1/1, 50 °C): δ 125.1 (C-2), 111.1 (C-3), 127.1 (C-3a), 120.0 (C-4), 120.8 (C-5), 130.7 (C-6), 112.6 (C-7), 137.3 (C-7a), 34.6 (Ar–6-CH_2), 41.8 (CH_2N), 33.8 (Ar–3-CH_2), 182.3 (COOH). UV (ethanol/water, 1/1) λ (log ε) 227 (4.47; maximum), 275 (3.63; shoulder), 279 (3.64; used for quantification of bound **5B** in **10B** and **11B**), 283 (3.68; maximum), 290 (3.61; shoulder). HRMS *m*/*z* 219.1173 ([MH]$^+$, $C_{12}H_{15}N_2O_2$ requires 219.1134), 202.0884 ([MH−NH_3]$^+$, $C_{12}H_{12}NO_2$ requires 202.0868), 156.0850 ([MH−NH_3−HCOOH]$^+$, $C_{11}H_{10}N$ requires 156.0813).

4.2. Protein conjugates (Fig. 4)

4.2.1. *S*-Acetylmercaptosuccinylated bovine serum albumin (8). To a solution of bovine serum albumin (Sigma, RIA grade; 200 mg, 2.99 μmol) in 0.125 M Na–phosphate buffer, pH 7.0, a solution of *S*-acetylmercaptosuccinic anhydride (60 mg, 345 μmol) in

N,*N*-dimethylformamide (0.2 mL) was added and the mixture was stirred under N_2, at room temperature, for 30 min, keeping the pH adjusted to 7.00.[43] The resulting solution was immediately passed through a column (37 × 2.5 cm) of Sephadex G-50 fine equilibrated and eluted with 0.2% NaCl to yield the modified protein (217.1 mg of lyophilized powder containing 40 mg of NaCl), which was stored desiccated at 4 °C. To estimate the number of *S*-acetylmercapto groups introduced, a stock solution of the freeze-dried material (15.3 mg corresponding to 12.5 mg of **8**) in 0.1 M Na–phosphate buffer (10 mL), pH 7.00, containing 2.5 mM of EDTA was prepared and aliquots (0.4 mL) were mixed with hydroxylamine stock (0.1 mL) and deacetylated for 30 min at room temperature to yield **9**. The crude reaction mixture was diluted with 0.1 M Na–phosphate buffer (2.5 mL), pH 8.0 containing 1 mM EDTA and 0.04 mL of Ellman's reagent were added. The number of thiol groups calculated from the absorbance at 412 nm was 16 per molecule of **9**.

4.2.2. Bovine serum albumin conjugate 10A. To a stirred solution of **5A** (5.5 mg, 25.2 μmol) in 0.1 M Na–phosphate buffer, pH 7.0, (0.5 mL) was added a solution of 4-(maleimidomethyl)-cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester (4.5 mg, 13.5 μmol) in THF (0.4 mL). The clear solution was stirred at room temperature for 6 h, until TLC (solvent A) indicated complete consumption of the *N*-hydroxysuccinimide ester (R_F = 0.8). The organic solvent was then removed in a stream of N_2, to yield a solution of crude 5-{2-[4-(maleimidomethyl)-cyclohexane-1-carboxamido]ethyl}indole-3-acetic acid (**6A**) (R_F = 0.45, solvent A_1), unreacted **5A**, and *N*-hydroxysuccinimide (R_F = 0.30, solvent A_1) in the above aqueous buffer.

Hydroxylamine stock was diluted with a 4-fold volume of 0.1 M Na–phosphate buffer, pH 7.0, and an aliquot (0.4 mL) was incubated with modified protein **8** (21.4 mg excluding NaCl, 0.32 μmol) for 30 min. The mixture was diluted with 0.1 M Na–phosphate buffer, pH 7.0, containing 2.5 mM EDTA (3 mL). An Ellman test performed with aliquots (2 × 50 μL) of this solution confirmed near-quantitative *S*-deacetylation to **9**. The remaining solution was immediately added to the reaction mixture containing **6A**, and the resulting clear solution was stirred at room temperature for 30 min. TLC (solvent A_1) indicated continuous presence of an excess of **6A**. The SH-groups of **9** completely reacted within 20 min, as revealed by Ellman assay. The protein conjugate (**10A**) formed was separated from small molecules on a column (37 × 2.5 cm) of Sephadex G-50 fine, equilibrated and eluted with 0.1 M Na–phosphate buffer, pH 7.0, containing 6 M urea, using UV absorbance (279 nm) and a Bradford test for detection. The respective fractions (elution volume ~50 mL) were desalted by ultrafiltration (AMICON DIAFLO YM 10; exclusion limit: 10,000 Da), by taking them to an approximate volume of 5 mL, diluting with redistilled water to 25 mL and repeating the ultrafiltration step. After eight such cycles the slightly opalescent solution was lyophilized to yield 19.2 mg of the pure protein conjugate. The number of residues of **5A** introduced was 16 per molecule of protein.

4.2.3. Bovine serum albumin conjugate 10B. In analogy with the preparation of **10A**, compound **5B** (5.4 mg, 24.8 μmol) was reacted with 4-(maleimidomethyl)-cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester (4.4 mg, 13.2 μmol) and the product was coupled to **9** [prepared from **8** (21.4 mg, 0.32 μmol)]. Purification of the conjugate was carried out essentially as described above. However, in the ultrafiltration step, 20 mM Na–phosphate buffer, pH 7.0, was used instead of the redistilled water to yield conjugate **10B** as lyophilized powder (33.1 mg) containing Na–phosphates (12.4 mg). The number of residues of **5B** introduced was 12 per molecule of protein.

4.2.4. Bovine serum albumin conjugate 11A. A solution of 4-(maleimido)butyric acid *N*-hydroxysuccinimide ester (3.5 mg, 12.5 μmol) in THF (0.3 mL) was added to a solution of **5A** (5.5 mg, 25.2 μmol) in 0.1 M Na–phosphate buffer, pH 7.0 (0.5 mL) and the mixture (clear yellowish solution) was stirred at room temperature for 1 h, until TLC (solvent A_1) indicated complete consumption of the *N*-hydroxysuccinimide ester (R_F = 0.80) and concomitant formation of coupling product **7A** (R_F = 0.40) and free *N*-hydroxysuccinimide (R_F = 0.30). Further binding of **7A** to **9** [from **8** (21.6 mg, 0.32 μmol)] and purification of the conjugate was performed as described for **10B**. The yield, after freeze drying, was 21.7 mg, 3.2 mg of which were buffer salts. The number of residues of **5A** introduced was 11 per molecule of protein.

4.2.5. Bovine serum albumin conjugate 11B. In analogy to the preparation of protein conjugate **11A**, 4-(maleimido)butyric acid *N*-hydroxysuccinimide ester (3.4 mg, 12.1 μmol), dissolved in THF (0.3 mL), was added to a solution of **5B** (5.6 mg, 25.7 μmol) in 0.1 M Na–phosphate buffer, pH 7.0 (0.5 mL). The product was coupled to **9** [from **8** (21.5 mg, 0.32 μmol)] to yield, after purification and freeze drying, 31.9 mg, 12.3 mg of which were buffer salts. The number of residues of **5B** introduced was 16 per molecule of protein.

4.3. Biotin conjugates (Fig. 5)

4.3.1. 6-{2-[6-(Biotinamido)hexanamido]ethyl}indole-3-acetic acid (13A). A suspension of compound **5B** (21.83 mg, 100 μmol), reagent **12A** (50.00 mg, 110 μmol), and 4-methylmorpholine (23.09 μL, 210 μmol) in anhydrous *N*,*N*-dimethylformamide (20 mL) was stirred overnight at room temperature. The resulting clear solution was evaporated in vacuo, at 35 °C; residues of *N*,*N*-dimethylformamide were removed by co-evaporation with toluene (4 × 5 mL). The remaining yellowish solid (81.6 mg) was extracted with EtOAc (6 × 2 mL) to remove most of the *N*-hydroxysuccinimide formed as a by-product. The dried residue was dissolved in 40% aq MeOH and passed through a disposable reversed-phase column (Varian Mega BondElut C-18, 10 g) previously rinsed with hexane (30 mL), EtOAc (30 mL), MeCN (30 mL), and MeOH (30 mL) and equilibrated with 40% aq MeOH. Fractions (10 mL)

were collected using the following sequence of eluents: 40% MeOH (10 mL effluent + 4 × 10 mL), 43% MeOH (5 × 10 mL), 46% MeOH (5 × 10 mL), 50% MeOH (5 × 10 mL). The title compound eluted with 43% MeOH, fr. 5, and 46% MeOH, fr. 1–5. Evaporation afforded a colorless solid (29.1 mg). The product was pure by TLC (R_f = 0.7, solvent A_2; 0.3, solvent B) except for very minor (<1%) amounts of high-R_f contaminants which, according to their color with the dimethylaminocinnamaldehyde and Ehrlich reagents, contain a biotin, but no indole, moiety. HRMS *m/z* 558.2714 ($[MH]^+$, $C_{28}H_{40}O_5N_5S$ requires 558.2750). MS/MS(558) *m/z* 512.2653 ($[MH-HCOOH]^+$, $C_{27}H_{38}N_5O_3S$ requires 512.2695), 340.1707 ([MH−6(2-aminoethyl)indole-3-acetic acid]$^+$, $C_{16}H_{26}N_3O_3S$ requires 340.1695), 227.0828 ([biotinoyl]$^+$, $C_{10}H_{15}N_2O_2S$ requires 227.0854), 202.0847 ($[MH-biotinoyl-Ahx-NH_2]^+$, $C_{12}H_{12}NO_2$ requires 202.0868).

4.3.2. 6-{2-[6-[6-(Biotinamido)hexanamido]hexanamido]-ethyl}indole-3-acetic acid (13B). A suspension of compound **5B** (9.26 mg, 42.44 μmol), reagent **12B** (26.50 mg, 46.68 μmol), and 4-methylmorpholine (9.80 μL, 89.12 μmol) in *N*,*N*-dimethylformamide (10 mL) was processed in essentially the same way as described for compound **13A**. The crude product (37.9 mg) was, after extraction with EtOAc (6 × 2 mL), dissolved in 40% aq MeOH and passed through a reversed-phase column conditioned as described above, using the following sequence of eluents: 40% aq MeOH (5 mL + 5 × 10 mL), 50% MeOH (5 × 10 mL), 60% MeOH (5 × 10 mL). The title compound eluted with 50% MeOH (fractions 4 and 5) and 60% MeOH (fractions 1 and 2). Evaporation afforded a white solid (21.7 mg, 76%) with the same degree of purity (R_f = 0.7, solvent A_2; 0.2, solvent B) as described for **13A**. HRMS *m/z* 671.3516 ($[MH]^+$, $C_{34}H_{51}O_6N_6S$ requires 671.3591). MS/MS (671) *m/z* 625.3536 ($[MH-HCOOH]^+$, $C_{33}H_{49}O_4N_6S$ requires 625.3536), 453.2562 ([MH−6-(2-aminoethyl)indole-3-acetic acid]$^+$, $C_{22}H_{37}N_4O_4S$ requires 453.2536), 340.1710 ([453.2536−$HN(CH_2)_5CO]^+$, $C_{16}H_{26}N_3O_3S$ requires 340.1695), 315.1712 ($[MH-biotinoyl-Ahx-NH_2]^+$, $C_{18}H_{23}N_2O_3$ requires 315.1709), 227.0857 ([biotinoyl]$^+$, $C_{10}H_{15}N_2O_2S$ requires 227.0854).

4.4. X-ray structure determination

Data collection was carried out on an Enraf Nonius CAD4 single crystal diffractometer at room temperature, using Cu K_α radiation. The crystal structures were solved by direct methods using the SHELX97[51] program. All nonhydrogen atoms were refined anisotropically by full-matrix least-squares calculations based on F^2 using the SHELXL97 program. The hydrogen atoms were treated using appropriate riding models and their coordinates were included in structure factor calculations. Crystallographic calculations and figure preparations were performed using the program package WINGX.[52] Crystallographic data (excluding structure factors) for the structures presented in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 246372 and CCDC 246373. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.5. Immunoassays

4.5.1. Antibody production. Monoclonal antibodies were raised in mice against bovine serum albumin conjugate **10B** at TNB Laboratories (St. John's, Newfoundland, Canada) according to the company's immunization protocol (http://www.mun.ca/seabright/tnb/monoclonal.html) and preselected based on their titer against the original antigen. The 'monoclonal antibody solutions' used in the assays described below were diluted (30 × in PBS), but were otherwise unprocessed supernatants from the antibody-producing hybridoma cultures.

4.5.2. Enzyme-linked immunosorbent assays. The experiments were performed in multiwell microtiter plates. Unless specifically indicated otherwise, all quantities indicated relate to a single well, and the wells were washed with TTBS (3 × 200 μL) between consecutive steps of the assay. The wells were first coated with goat antimouse IgG, γ-chain, by incubating (4 °C, overnight) with a solution (100 μL) of the immunoglobulin (0.02 mg/mL in 0.1 M carbonate buffer, pH 9.6). Remaining protein binding sites and exposed well surfaces were blocked (room temperature, 7 h) with 1% gelatin in TTBS (blocking buffer; 200 μL). An aliquot (100 μL) of the monoclonal antibody solution was then added, followed by incubation at 4 °C overnight. The immobilized antibodies were exposed (3.5 h, room temperature) to a series of dilutions (0.746 nM to 7.46 μM) of biotin conjugate **13A** or **13B** in blocking buffer using 100 μL aliquots of solution per well and four replicate wells for each dilution. The biotin moieties of the bound conjugates were coupled to streptavidin-linked alkaline phosphatase, adding aliquots (100 μL) of a diluted (1000 × in blocking buffer) commercial ('Streptavidin AP Conjugate', cat. no. 69219, CalBiochem, San Diego, CA, USA) preparation, followed by incubation (3 h) at room temperature. After washing with TTBS (3 × 200 μL) and TBS (2 × 200 μL), the bound enzyme was detected by its hydrolytic activity, applying an aliquot (200 μL) of *p*-nitrophenylphosphate solution (1 mg/mL) in 0.1 M carbonate buffer, pH 9.6. After incubation (37 °C, 2 h) the amount of *p*-nitrophenol formed was determined from its absorbance at 405 nm.

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