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IMMUNOASSAY OF PODOPHYLLOTOXIN

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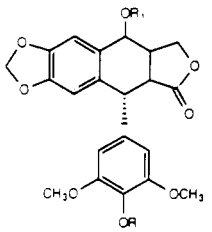
ABSTRACT.—The purpose of our research was to design and develop a sensitive immunoassay for the natural *Podophyllum* lignan, podophyllotoxin [**1**], which is the precursor of the anti-cancer drugs etoposide [**2**] and teniposide [**3**]. In order to obtain a specific antiserum, bovine serum albumin and keyhole limpet hemocyanin conjugates of succinyl-podophyllotoxin [**5**] were prepared as antigenic hapten carrier proteins. These antigens were used to develop polyclonal antibodies to **1** in rabbits. The antisera were used for the development of an immunoassay for the detection of **1** and its 4′-demethyl derivative **4**. As little as 0.16 nmol of **1** is detectable with this ELISA. The assay was applied to the direct analysis of **1** in plants for the screening of potential drug plants which allowed quantitation in less than 0.2 mg of plant tissue.

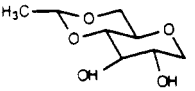
Etoposide [**2**] and teniposide [**3**] are semisynthetic podophyllotoxin [**1**] derivatives which have a wide spectrum of antitumor activity (1) and have been synthesized from **1** (2,3). The availability of the precursor has diminished due to declining populations of the source plants (4). In addition, in the clinical use of these drugs for the control and cure of cancer it has been shown that these drugs are enzymatically degraded to **1**, with toxic side effects (5). Several radioimmunoassay (RIA) methods have been developed for the quantitation of **2** and its analogues in human plasma (6–8), but not for **1**. Therefore, a rapid, sensitive, and inexpensive technique for quantification of **1** in plants and human plasma is highly desirable.

RESULTS AND DISCUSSION

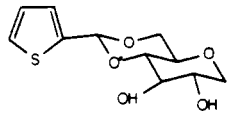
The modification of **1** (300 mg, 0.72 mmol) with succinic anhydride (313 mg,



1 R=Me, R_1=H

2 R=H, R_1= 

3 R,R_1=H

4 R=H, R_1= 

5 R=Me, R_1=$COCH_2CH_2COOH$

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3.14 mmol) in dry pyridine resulted in the succinylation at the 4 position of the central saturated ring of **1**. Compound **5** (36.6 mg) was purified by Sephadex LH-20 cc and analyzed by tlc, ir, nmr, ms, and elemental analysis. Tlc R_f 0.35; ^{1}H nmr ($CDCl_3$) δ 1.70 (1H, H-2), 2.65 (4H, -OCH_2CH_2CO-), 2.78 (1H, H-3), 3.71 (9H, 3 × -OMe), 4.15 (1H, H-4), 3.95 (2H, Hα-2a and Hβ-2a), 4.15 (1H, H-1), 5.91 (2H, -OCH_2O-), 6.05 (2H, H-2′ and H-6′), 6.58 (2H, H-5 and H-8); ir 3412, 2940, 1745 cm^{-1}; ms $[M]^+$ 410 (100%), 395, 380, 167, 149, 101, 57; elemental analysis C 60.74%, H 5.52%.

The immunogenic antigens **5**-BSA (bovine serum albumin) (21.5 mg) and **5**-KLH (keyhole limpet hemocyanin) (30.2 mg) were prepared by coupling the hapten to free amino groups of the proteins by the mixed anhydride reaction. Coupling ratios were 27.8 and 8.8 mol of **5** per mol of BSA and KLH, respectively, as determined by spectroscopic analysis [**5**: $\lambda_{max} = 260$ nm, $\epsilon = 1.29 \times 10^5$ $cm^2 \cdot mol^{-1}$; molar coupling ratio = $(\epsilon_{conjugate} - \epsilon_{protein}) = \epsilon_5$]. The difference between the mol wt of the conjugates and those of the carrier proteins also were determined by SDS-PAGE analysis. SDS-PAGE analysis showed that 39.8 molecules of **5** bound to one molecule of BSA. KLH conjugates could not be analyzed by electrophoresis due to the aggregation of KLH protein; this resulted in several bands in the gel.

These antigens were used to develop polyclonal antibodies to **1** in rabbits for the development of an immunoassay for the quantitative analysis of **1** and **4** and adaptation of the ELISA technique for detection of these molecules in plants. Both immunogens gave positive results in a preliminary check of antibody titer and selectivity, but the titer achieved in response to **5**-KLH was poor. A standard curve for **1** was obtained by plotting absorbance of the reaction well versus the concentration of each compound tested (0.078–5 μg or 0.18–12.0 nmol) (Figure 1).

Compounds **2** and **4** cross-reacted with the anti-**1** antiserum, as shown in Figure 2. Because **2** and **4** cross-react, it can be assumed that the antigenic determinant is neither the hydroxyl group of **4** nor the ethylidene glucosidic moiety of **2**. It can be speculated therefore that the main antigenic determinants are regions other than these two sites. However, the lesser reactivity with **2** may indicate that the substitution sterically hinders binding between the antigen and at least some of the antibodies present.

RIA techniques for measuring the epipodophyllotoxin derivatives **2** and **3** have been reported (7,8), but no RIA technique has been reported for **1**. Compound **1** is the

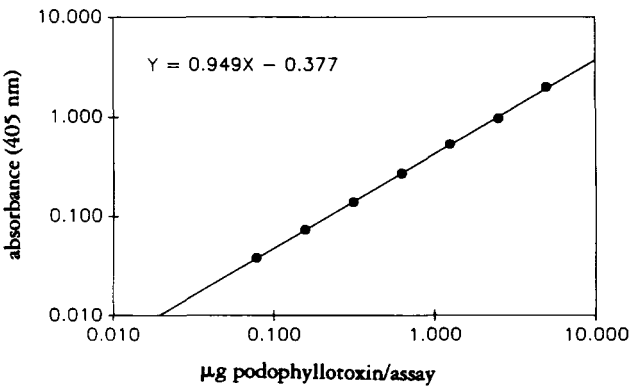

FIGURE 1. Standard curve of the ELISA assay for **1** ($n = 9$, $R^2 = 0.998$). Error bars in all figures are standard deviations of the means. Where not shown, error bars were less than the symbol size.

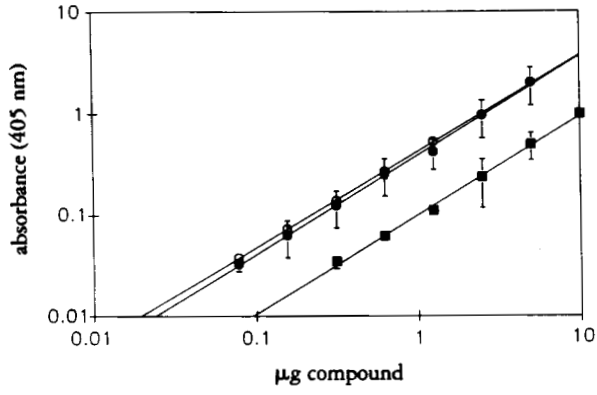

FIGURE 2. Cross-reactivity of **1** and its derivatives. ○, **1**; ●, **4**; ■, **2**. All samples were run in duplicate (●, ■) or triplicate (○).

precursor compound and the most active cytotoxic metabolite in vivo of these drugs. RIA combines the specificity of antibody-antigen binding with the sensitivity of radioactive trace measurement. The limit of detection with this technique is in the range 0.025–5 μg (0.042–8.49 nmol) of **2**. Although less sample is required in RIA, its major drawbacks are the use of radiotracers and expense.

An ELISA for **1** utilizes the unique specificity of antigen-antibody binding and the simplicity of an enzyme activity reaction. The immunoassay was highly sensitive for a dilution series of crude H_2O and EtOH extracts of *Podophyllum peltatum* L. (Berberidaceae). A comparison of the plant extract dilution curves with the standard curve is shown in Figure 3. It should be noted that serial dilutions of the plant extracts of *P. peltatum* covering the entire range of the standard curve yielded values exactly paralleling those of the standard curve. This parallelism test demonstrated that a linear relationship exists between the extract volume used for immunoassay and the **1** value measured; thus, from each diluted sample the same concentration of reactive material within the plant could be calculated. The minimum amount of *P. peltatum* root or rhizome material needed for a positive assay was calculated to be less than 0.2 mg. The minimum amount of **1** detected by the ELISA technique was calculated to be 0.16 nmol (0.067 μg).

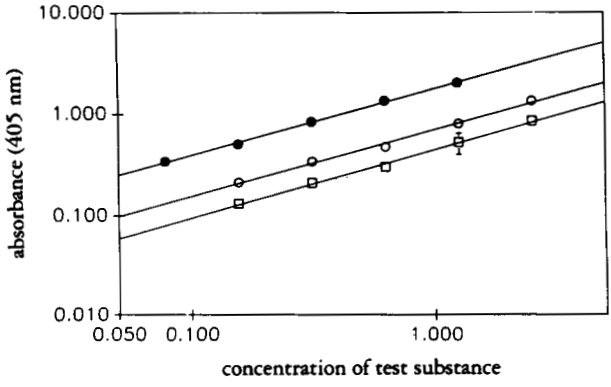

FIGURE 3. Parallelism of plant extract dilution curves and standard curves. ●, standard curve (μg **1**/well); ○, EtOH extract (mg plant/well); □, H_2O extract (mg plant/well). For the experiment, crude EtOH and H_2O extracts of *Podophyllum peltatum* were used.

The assay was also applied to the direct analysis of the distribution of **1** in crude extracts of various organs of *P. peltatum*. Low concentrations in some organs (pedicels, petioles, and pericarps) required the use of a standard addition technique. The concentrations of **1** in these organs descended in the order roots (11.5 mg/g)>rhizomes (0.37 mg/g)>leaves (0.088 mg/g)>pericarps (0.0093 mg/g)>petioles (0.00887 mg/g)> pedicels (0.00285 mg/g). This may explain how this plant is protected from fungal infection and insect feeding. The central idea of plant defense (9) is that plant species evolve secondary compounds suich as lignin, alkaloids, tannin, phenols, and toxins in response to attacks by insects or microbes. A fungus, *Septotinia podophyllina*, infects *P. peltatum* and causes a leaf blotch of the plant (10). Individual leaves and stems of this plant live only a season, but the underground roots and rhizomes live for decades to hundreds of years. It is quite likely that the leaves and stems, which possess a small amount of the toxin, would be more heavily damaged, but it is clear that roots and rhizomes are difficult for fungi to infect, thus ensuring survival of the plant. Since the amounts of **1** in the roots are more than 100 times, and those of the rhizomes at least four times, that of the leaves, the roots and rhizomes are protected even while leaf tissue may be subject to greater damage. These ideas could be tested by measuring concentrations of **1** in young plants, more subject to fungal attack (11), and by quantitative analysis of the extent of the damage to above- and below-ground organs during the growing and dormant seasons.

In order to confirm the validity of the new ELISA method, quantitative comparison was made between the ELISA and the conventional hplc method (12) for determination of **1** (Figure 4). The concentrations determined by both the hplc and ELISA methods agreed closely for the roots, rhizomes, and leaves. The reason for the low agreement be-

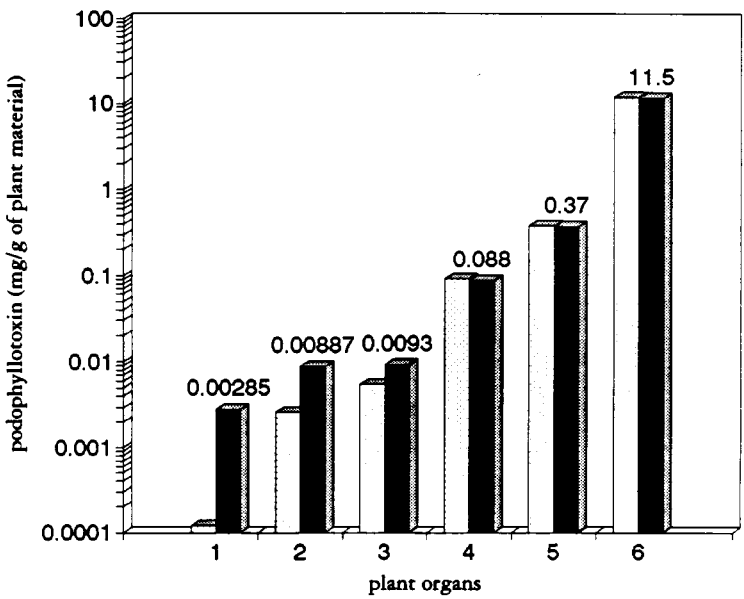

FIGURE 4. Hplc (▥) and ELISA (■) determination of **1** in different organs of *Podophyllum peltatum*. The value above each pair of bars is the concentration of **1** determined by the ELISA procedure. Hplc values: 1 = pedicel (0.00012 mg/g of dry wt); 2 = petiole (stem) (0.00262 mg/g); 3 = pericarp (0.0055 mg/g); 4 = leaf (0.092 mg/g); 5 = rhizome (0.386 mg/g); 6 = root (11.758 mg/g).

tween the two methods for the organs of low concentration, pericarps, petioles, and pedicels, is that the values were obtained by standard addition for both methods. Eighty to 100 times more **1** was added to an ELISA well to give a suitable absorbance than was necessary for the hplc analysis. The values of **1** detected in the pericarps, petioles, and pedicels by the ELISA method therefore have larger errors associated with them. This suggests that hplc is more appropriate for extremely low concentrations of **1**. The ELISA method is much faster to perform than hplc analysis. Improvements in sensitivity could possibly be obtained using RIA or chemoluminescent modification. Attempts to improve sensitivity using a avidin-biotin complex technique led to no improvement (data not shown).

The main purpose of this research was to adapt the ELISA technique for detecting **1** in source plants; it is most advantageous and a faster method where a large number of samples need to be analyzed. Since the final step is a color intensity measurement, ELISA offers the potential of being used for a quick field determination of **1**. Although the assay was highly reliable and reproducible with pure standard solutions, the anti-**1** antiserum had cross-reactivity with structurally similar compounds **2** and **4**. However, α-tetralol, a compound structurally related to the central rings of **1**, did not cross-react with this antibody. The anti-**1** antiserum did not cross-react with a plant alkaloid with similar antimitotic activity, colchicine; two chemotherapeutic drugs, vinblastine, vincristine, or the antibiotic novobiocin (10 μg each were tested), which could be used in chemotherapy regimens. This suggests that this ELISA may also be appropriate for clinical pharmacokinetic studies of the semisynthetic derivatives **2** and **3**, even in patients who are also receiving other primary chemotherapy. Further studies using the assay described in this paper should be applied to clinical serum samples.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Unless otherwise stated, reagents were of the highest grade available. Immunochemicals were obtained from Pierce (Rockford, IL), and etoposide [**2**] and podophyllotoxin [**1**] were received as gifts from Bristol-Myers Squibb Co. (Wallingford, CT). Compound **4** was synthesized from **1** using trimethylsilyliodide (13). All other reagents were obtained from Sigma (St. Louis, MO).

The hapten was synthesized by adding succinic anhydride to **1** in 5 ml of $CHCl_3$ containing 1 ml of dry pyridine. The mixture was refluxed for 5 h; this was determined experimentally to give a maximal yield of final product with minimal side products. The reaction was monitored by using Si gel tlc plates developed in a solvent system of $CHCl_3$-MeOH (90:10) for a distance of 5 cm followed by $CHCl_3$-Me_2CO (65:35). Purification of the reaction mixture by Sephadex LH-20 cc using $CHCl_3$–*n*-hexane–MeOH (5:5:1) gave **5** as a single compound. This compound was characterized by tlc, ir, nmr, ms, and elemental analysis.

The hapten **5** was coupled to free amino groups of the carrier proteins, BSA or KLH, via a mixed anhydride reaction (14). Compound **5** (16 mg, 31.1 μmol) and 75 μl tri-*n*-butylamine were dissolved in 2.5 ml DMF and cooled to 0°. To this solution, 20 μl isobutylchlorocarbonate was added, and the formation of the mixed anhydride was allowed to proceed for 20 min. The solution was then added to an ice-cold solution of 40 mg of BSA or KLH in 22 ml of DMF-H_2O (1:1) with thorough stirring. The mixture was stirred for 3 h and then dialyzed against 10% DMF in distilled H_2O. Characterization was done using spectroscopy (Perkin Elmer Lambda 6, Norwalk, CT) (15) and electrophoresis.

The injections of antigens, maintenance of rabbits, and collection of sera were contracted to Hazleton Research Products, Inc. (Denver, PA). The antigens, **5**-BSA and **5**-KLH, were injected nodally into rabbits at multiple sites with the same amount of complete Freund's adjuvant. Two i.d. booster injections were made in the back and sides at four-week intervals. Ten days after the final booster, blood was collected and sera were tested for antibody. The antibodies were isolated by precipitation with 45% saturated $(NH_4)_2SO_4$ (16). The precipitate was pelleted by centrifugation (10,000 × *g*, 30 min, 4°). The pellet was resuspended in phosphate-buffered saline (pH 7.4) and dialyzed, lyophilized, and stored at −70°.

An antibody-sandwich ELISA procedure based on that of Ausubel *et al.* (17) was used for detecting the antigen; this procedure is summarized in Scheme 1. Antisera were used in the appropriate dilution as determined by criss-cross assay (18). The dilutions of standard and tracer were made using phosphate-buffered saline (BupH™ Dulbecco's PBS: 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14

Coating

Fill each well of the 96-well ELISA plate with 100 µl of the diluted antibody and incubate overnight at 4°.

↓

Wash

Empty antibody, block, and fill each well with 100 µl of standard or antigen and incubate 1 h at room temperature.

↓

Wash

Add 100 µl of the capture antibody and incubate overnight at 4°.

↓

Wash

Incubate with antibody-enzyme conjugate for 2 h at room temperature.

↓

Wash

Add substrate; stop reaction after 30 min with 3 M NaOH.

↓

Read at 405 nm

SCHEME 1. Flowchart of antibody-sandwich ELISA procedure.

M NaCl, 0.01 M KCl, pH 7.4). Coating of the plates with dilute antibody (1.25 µg/ml, 100 µl; coating buffer 0.2 M sodium carbonate-bicarbonate buffer, pH 9.4) was done overnight at 4°. After blocking the remaining surfaces with 1% BSA in PBS for 1 h at room temperature, 100 µl of the standard (10 µg/ml) or sample was added and incubated for 1 h at room temperature. After washing the unbound antigen with washing buffer (0.1% BSA in PBS), the second addition of the primary antibody was made and incubated for overnight at 4°; this second application serves as a capture antibody. The diluted secondary antibody-enzyme tracer (goat anti-rabbit IgG conjugated with alkaline phosphatase, 100 µl, 1:3200 dilution) was subsequently added for 2 h at room temperature. The solutions were then drained on a paper towel and the plates were rinsed three times with 100 µl washing buffer (0.1% BSA in PBS).

The quantity of surface-bound secondary antibody was measured by assaying alkaline phosphatase activity. A 100-µl aliquot of a 1 mg/ml solution of *p*-nitrophenyl phosphate substrate was added. The enzyme reaction proceeded at room temperature for 30 min and was stopped with 100 µl 3 M NaOH. The amount of *p*-nitrophenyl phosphate substrate hydrolyzed, which is directly related to the amount of the secondary antibody bound to the primary antibody, and so proportional to the amount of antigen present, was measured on a manual microtiter plate reader (Danatech Minireader II, Chantilly, VA) at 405 nm. Each assay was replicated three times for each sample. A standard curve was constructed from the data produced by serial dilutions of the standard antigen. Cross-reactivity tests were done using compounds **2**, **4**, α-tetralol, and chemotherapeutic agents of unrelated structure.

P. peltatum was collected from the wild, and the various organs were separated and dried. A voucher specimen has been retained in the herbarium of the Philadelphia College of Pharmacy and Science, Philadelphia. Each sample was ground to pass a 20 mesh screen, and subsamples (500 mg) were extracted in 10 ml of EtOH. The solvent was removed by filtration and the residue was further extracted in 10 ml of H_2O, which was also filtered. The filtered extracts were collected, the solvents removed by rotary evaporation, and the residue taken up in PBS for use in the assay. Each 100 µl of undiluted plant extract sample in PBS corresponds to 5.0 mg of plant material. Each extract was serially diluted for determination of the parallellism of the assay with the standard curve.

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