Lack of Specificity of Current Anti-Digoxin Antibodies, and Preparation of a New, Specific Polyclonal Antibody That Recognizes the Carbohydrate Moiety of Digoxin

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Current immunoassays for digoxin do not distinguish digoxin from its glycosidic metabolites. We have synthesized a novel digoxin/bovine serum albumin conjugate via reductive ozonolysis of the lactone ring such that the carbohydrate moiety of digoxin remains intact. Antibodies raised against this conjugate show minimal cross reactivity to digoxigenin, bisdigitoxide, monodigitoxide, digoxigenin, and digitoxin. With this antibody, digoxin can be measured in the presence of these metabolites.

The cardiac glycoside digoxin is one of the most commonly prescribed drugs. Despite the vast literature on its use to treat cardiac irregularities, the pharmacological basis of its action in relation to its structure remains poorly understood. The biological activity of digoxin and its analogs is conveniently measured in vitro via the inhibition of ATP-dependent cation uptake in erythrocytes (1, 2), although this has yet to be proven to be an accurate measure of cardiac efficacy. The measurement of digoxin concentration is complicated by its metabolism in vivo. Digoxin is a steroid–carbohydrate conjugate (Figure 1). Although there is considerable variation among patients, a major route of metabolism is the sequential loss of glycosidic units or saturation of the steroid lactone ring—or both (3–4). The resulting metabolites retain various degrees of the biological (5, 6) and toxic (7) activities of native digoxin. Moreover, these metabolites may interfere with the accurate radioimmunoassay of digoxin (8, 9). Digoxin concentrations in serum are routinely measured by immunoassay with an antibody to digoxin raised against a conjugate of bovine serum albumin (BSA) and digoxin. This conjugate is prepared by periodate oxidation of the vicinal hydroxyl groups of the terminal sugar and coupling the so-generated aldehyde groups to the amino groups of BSA (10). Thus, the conjugate linkage is through the carbohydrate moiety of digoxin. The antibodies generated against this conjugate are, therefore, for the most part directed against the steroid moiety of digoxin. As a result, digoxigenin bis- and monodigitoxides, and digoxigenin itself, all react with the antibodies, whereas dihydrodigoxigenin and dihydrodigitoxin—metabolites in which carbon 22 is reduced—show little or no cross reactivity. The cross reactivity of the metabolites of digoxin with the anti-digoxin antibodies is well known, and for most commercial anti-digoxin sera the degree of cross reactivity is stated. Indeed, for some antisera, the carbohydrate-deficient metabolites are more potent antigens than is digoxin itself, the additional carbohydrate units of digoxin in some way reducing the antibody-binding activity.

There are considerable variations in the reports of the degree to which the glycosidic metabolites may be detected as apparent digoxin concentrations in serum samples. Most reports indicate minor metabolism of the carbohydrate (9, 13). However, Peters et al. (3), monitoring digoxin metabolism in 100 patients, found that the glycosidic metabolites accounted for an average of 20% of the total digoxin concentration. Digoxin and its metabolites can be separated by "high-performance" liquid chromatography before RIA (9). Moreover, there are many reports of naturally occurring endogenous digoxin-like-factors, defined by their cross reactivity in the RIA for digoxin (11–14). Such species have not been characterized further, but may interfere with immunoassay of serum digoxin (14).

Because of the problems in the measurement of digoxin in serum, we undertook a detailed study of the specificity of the RIA for digoxin and examined the reactivity of not only the metabolites but also some 30 additional cross-reactive, often unrelated, compounds (15). We suspected that the cross reactivity, at least with the metabolites, might be ascribed to the nature of the original immunogen, i.e., a result of the method used to conjugate BSA to digoxin. In the preparation of a digoxin conjugate via periodate oxidation of digoxin, the immunogenic determinants that might elicit antibodies capable of distinguishing digoxin from the bis- and monodigitoxides or digoxigenin are either destroyed (the terminal sugar is oxidized) or may be masked by the protein. Given the uniqueness of the carbohydrate moiety of digoxin, we thought that an antibody raised against this region of the digoxin molecule might be more specific for digoxin. To raise such antibodies, we reasoned that a method was needed for conjugating digoxin to a carrier protein without modifying the carbohydrate moiety. In this paper, we present a method for coupling digoxin via the lactone ring, and we describe the use of this conjugate to raise a carbohydrate-specific antiserum that can distinguish between digoxin and its glycosidic metabolites. We determined the specificity of this antiserum and compared the results of immunoassay of digoxin in the presence of the metabolites by use of this antiserum and a commercially available one.

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**Materials and Methods**

Purification of anti-digoxin antibodies from commercial immune sera. Anti-digoxin antibodies raised against digoxin conjugated to BSA by periodate oxidation were purchased from Antibodies Inc., Davis, CA, and Wien Laboratories, Succasunna, NJ. Anti-digoxin activity was determined by using an \(^{3}H\)-labeled digoxin RIA as previously described (15). The immune serum was precipitated with 40%-saturated ammonium sulfate. The precipitate was redissolved in, and dialyzed against, 0.1 mol/L phosphate-buffered isotonic saline, pH 7.2. Anti-digoxin antibodies were further purified by immunoaffinity chromatography with use of digoxin covalently linked to agarose by a photoactive hetero-bifunctional crosslinking agent (16). We treated 3 mL of amine-activated agarose beads with 10 mL of hydroxysuccinimidyl azidobenzoate (2 mMol/L) in the dark for 1 h at room temperature. After the beads were washed with water, we added 2 mg of \(^{3}H\)digoxin in 2 mL of an equivalent mixture of ethanol and water and removed the solvent by rotary evaporation, during which the digoxin was adsorbed onto the beads. The dry beads were then irradiated, with stirring, for 2 min, 1 cm from an ultraviolet source; this covalently attached the adsorbed digoxin to the agarose beads. The beads were washed successively with 100 volumes of the ethanol/water mixture and 50 volumes of water. After concentrating the wash fractions to 2 mL, we measured the unbound \(^{3}H\)digoxin and calculated the amount of digoxin coupled to the agarose (0.2 \(\mu\)mol/mL of beads).

The immunoglobulin fraction of 1 mL of immune serum was applied and the digoxin column was washed with phosphate-buffered saline. Anti-digoxin activity was measured by the \(^{125}\text{I}-\text{RIA} method (15). All anti-digoxin activity was bound to the column. Specific antibodies were eluted by adding 10 mL of a 1 mol/L solution of KI, then 10 mL of a 1 mol/L solution of potassium persulfate. To this solution we added 2 mL of ethanol, 0.1 mol/L pyridine, and 1.0 mL of acetic acid (17). The eluted fractions were pooled, dialyzed, concentrated against sucrose, again dialyzed to remove the sucrose, and used in the RIA.

**Reductive ozonolysis.** We dissolved 128 \(\mu\)mol of digoxin (Sigma Chemical Co., St. Louis, MO) together with 2.5 \(\times\) 10\(^{7}\) dpm of \(^{12}\text{H}\)digoxin (91 Ci/mol; New England Nuclear, Boston, MA) in 3.5 mL of dichloromethane/methanol (1:1, by vol). The solution was cooled to \(-78^\circ\text{C}\) in a solid CO\(_2\) slush in acetonitrile. Ozone from a high-voltage ozone generator was bubbled through the solution at 0.4 mL/min in the presence of a starch/iodine paper. After 5 min, the tube was capped and the reaction mixture was stirred for 3 h at \(-78^\circ\text{C}\). We then flushed the reaction tube with nitrogen, added 2 mL of dimethyl sulfoxide (Sigma), and stirred the mixture overnight in a Dewar flask, allowing the temperature gradually to rise to 25°C. The solvent was then evaporated under nitrogen.

**Coupling to methylated BSA (mBSA).** We dissolved 30 mg of mBSA in 12.5 mL of dichloromethane/methanol (2:3, by vol) and adjusted the pH to 11 with 0.1 mol/L NaOH. We dissolved in this solution the viscous product obtained after reductive ozonolysis, stirring this reaction mixture at room temperature overnight. We then added 128 \(\mu\)mol of sodium cyanoborohydride and stirred the conjugation reaction for another 4 h at room temperature. The reaction mixture was dialyzed vs water for four to six days, after which the conjugate was lyophilized or stored at \(-20^\circ\text{C}\) prior to immunization.

**Alkaline hydrolysis.** After reductive ozonolysis, we treated the reaction mixture with 0.1 mol/L KHCO\(_3\) for 3 h at room temperature. The reaction product was separated by preparative "high-performance" thin-layer chromatography in ethylacetate/acetone (2:1, by vol). We scraped and eluted the product from the silica gel plate and subjected it to nuclear magnetic resonance and mass spectral analyses.

**Nuclear magnetic resonance analysis.** We concentrated the sample to dryness three times by flash evaporation from 95.5% deuterium oxide and dissolved it in pyridine for analysis. The spectra were taken with a Nicolet 360-MHz spectrometer, with quadrature detection in the Fourier transformation mode at 25°C. Acetone was used as the internal standard. Individual peaks were assigned by decoupling.

**Gas chromatograph–mass spectrometric analysis.** After reductive ozonolysis and alkaline hydrolysis, we reacted the digoxin derivative with heptasfluorobutyric anhydride as previously described for digoxin (17). To analyze the product, we used a Hewlett-Packard 5988B gas chromatograph–quadrupole mass spectrometer operating in the electron impact mode and coupled to a Hewlett Packard 50-1000E series computer.

**Immunization.** We dissolved the conjugate (1 mg of protein containing \(-50\) digoxin molecules per molecule of BSA) in 1 mL of water, emulsified this with an equal volume of Freund's complete adjuvant, and injected aliquots subcutaneously into a rabbit at multiple sites. A booster injection of the same conjugate (1.5 mg of protein) in incomplete adjuvant was administered after one month. A second booster with a less potent conjugate (5.8 mg of protein containing \(-50\) digoxin molecules per molecule of BSA) was given three weeks later. We collected serum samples periodically and assessed these for anti-digoxin activity—undiluted before the second booster was given, and diluted 10-fold thereafter.

**\(^{3}H\)Digoxin radioimmunoassay.** The method we used was adapted from one previously described (18). Briefly, tubes containing 0–30 ng of \(^{3}H\)digoxin (3000 dpm) in 0.1 mol/L phosphate-buffered isotonic saline (pH 7.2) were incubated at room temperature for 15 min in the presence of diluted anti-digoxin antiserum. We added ice-cold dextran-coated charcoal and incubated the mixture at 4°C for 10 min. After centrifugation, we counted the radioactivity of an aliquot of the supernate in a liquid-scintillation counter to determine the unbound \(^{3}H\)digoxin. Controls (reagent blanks and reagent with nonimmune serum) were assayed in parallel.

**ELISA assay.** Each well of multi-well ELISA plates was coated with 500 ng of digoxin–BSA conjugate or BSA alone. Serial dilutions of immune vs nonimmune serum were assayed for antigen binding by using anti-rabbit Ig–horseradish peroxidase conjugate. Binding was quantitated with a Dynatech automatic ELISA reader.

**Results**

Specificity of Commercial Anti-Digoxin Sera

The structure of digoxin is represented in Figure 1. Cross reactivity of commercial anti-digoxin antibodies towards the digoxin metabolites in which one, two, and three sugars have been removed (digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin, respectively) was measured (Table 1). We found that these carbohydrate metabolites of digoxin are approximately three- to fourfold more antigenic than native digoxin in the standard assay for digoxin. The antiserum does not react with dihydridigoxin. Purification of the anti-digoxin antibodies by affinity chromatography considerably reduced the cross reactivity, such that the metabolites were now recognized on an equal (one-to-one) basis relative to digoxin. However, even the affinity-purified anti-digoxin antibodies failed to distinguish digoxin from the glycosidic metabolites.

Ozonolysis of Digoxin and Characterization of Products

The outline of the reaction scheme is depicted in Figure 2. We proposed to generate an aldehyde group (Figure 2C) by...
opening the lactone ring by reductive ozonolysis, and to couple this functional group to the amino groups of BSA. The products of reductive ozonolysis of digoxin were separated by thin-layer chromatography and made visible by a carbohydrate-specific staining procedure (Figure 3). Little or no starting material remained and a new, diffuse band of reduced mobility was detected (Figure 3, lane g). Although reaction with 2,4-dinitrophenyldiazine indicated the presence of a carbonyl group, we failed to find a significant signal by nuclear magnetic resonance analysis for the aldehydic proton expected for structure C in Figure 2. We speculated that if such a structure were formed, the ester would be unstable and spontaneously lose glyoxylate to form compound D (Figure 2). We therefore repeated the reaction and hydrolyzed the products under mild basic conditions (Figure 3, lane f).

The nuclear magnetic resonance spectra of the resulting derivative (presumably compound D) and that of native digoxin were compared (Figure 4). Several features of the spectra demonstrate that the lactone ring has been modified: the olefinic proton on C-22 has been completely removed, and the quartet of methylene protons at C-20 has moved upfield, indicating a change in electronic environment, whereas the C-18 methyl protons (but not the C-19 methyl protons) have shifted downfield. The proton on C-12 has shifted upfield and, surprisingly, the proton at C-3 is also shifted downfield in the hydrolyzed product. The changes in proton chemical shifts are summarized in Table 2. The signals from the carbohydrate protons before and after treatment are superimposable. Had glycosidic cleavage occurred, there would have been significant change in the carbohydrate proton resonance, particularly C-4, but this did not happen (Figure 4, Table 2). The 8-Hz coupling...
Table 2. Change in Proton Chemical Shifts after Ozonolysis and Alkaline Hydrolysis

| Proton \(|^a|\) | Digoxin | Compound D |
|--------------|---------|------------|
| C-22         | 6.325   | —          |
| anomic \(|^b|\) | 5.436   | 5.433      |
| (C-1', "")   | 5.410   | 5.407      |
| C-21         | 5.338   | 4.941      |
|              | 5.287   | 4.887      |
|              | 5.193   | 4.751      |
|              | 5.142   | 4.702      |
| C-3' """"c    | 4.682   | 4.689      |
|              | 4.654   | 4.651      |
|              | 4.481   | 4.474      |
| C-3, C-12    | 3.812-3.741 | 3.966-3.914 |
| C-12         | -       | 3.736-3.693 |
| C-4' """"c    | 3.625   | 3.620      |
|              | 3.596   | 3.590      |
|              | 3.566   | 3.558      |
|              | 3.513   | 3.509      |
|              | 3.486   | 3.483      |
| C-18         | 1.264   | 1.316      |
| C-19         | 0.877   | 0.865      |

\(^{a}\) Superscripts refer to protons of the carbohydrate moiety (see Fig. 4). \(^{b}\) The anomic protons of the three sugars are superimposed to give a doublet with an approximate 8Hz coupling. \(^{c}\) Small couplings on major peaks have been averaged.

for the anomic protons confirms the β linkage of the glucose moieties.

We also subjected "compound D" to mass spectral analysis (Figure 5) after cleavage of the carbohydrate moiety and derivatization with heptafluorobutyrate. The aglycone of compound D containing one heptafluorobutyrate group would give an ion peak at 596. Cleavage of the C-13-C-17 and C-14-C-15 bonds—as has been shown to occur for progesterone derivatives (29)—would result in a peak at 496. Loss of heptafluorobutyrate from this species would give a peak at 282, whereas loss of CO (at C-16) would give a signal at 468. Further loss of heptafluorobutyrate would give a peak at 254 or loss of CH₃ (at C-20) would account for the signal at 453 m/e. Thus, the major peaks observed (Figure 5) coincided with those predicted from structure D (Figure 2).

Coupling of Digoxin to mBSA

Nuclear magnetic resonance and mass spectral analyses confirmed the production of compound D after ozonolysis and alkaline hydrolysis of digoxin. It is therefore reasonable to assume that compound C (Figure 2) is the immediate product of reductive ozonolysis. Immediate formation of a Schiff's base with the aldehyde generated at C-23 should stabilize the ester linkage between C-23 and C-21. Our results suggest this to be the case. mBSA was added directly to the reaction mixture after the dimethyl sulfide reduction step, and the conjugated product was reduced with cyanoborohydride. Analysis of the reaction mixture by thin-layer chromatography revealed a new carbohydrate-containing species (Figure 6, lane e), which remained at the origin. Unconjugated mBSA stays at the origin but does not stain for carbohydrate (Figure 6, lane f). The reaction mixture was extensively dialyzed, removing the unconjugated steroid derivatives (Figure 6, lane g). We calculated the stoichiometry of conjugation from the amount of [³H]digoxin incorporated into the conjugate: between 40 and 50 digoxin molecules were coupled per mBSA molecule.

Immunization and Characterization of Antibody Specificity

We detected anti-digoxin approximately eight weeks after subcutaneous injection of the mBSA-digoxin conjugate (Figure 7). By the [³H]digoxin RIA, the percentage of digoxin

![Fig. 6. Thin-layer chromatography of reaction mixture after protein conjugation](image)

![Fig. 7. Immunization schedule and results](image)
bound was constant up to a 40-fold dilution of antibody; however, considerably greater reactivity was demonstrated in the ELISA assay (Figure 8). There was preferential binding to the mBSA–digoxin conjugate in which the carbohydrate moiety was preserved intact (compare parts a and b of Figure 8). Using this assay, we could detect activity binding above background for immune serum diluted 1 in 10⁶.

The degree of cross-reactivity of the digoxin metabolites was monitored with the [³H]digoxin RIA. The binding of [³H]digoxin was effectively displaced by unlabeled digoxin to give the standard curve shown in Figure 9. The bisdigotoxoside (Figure 9a) was a considerably less potent inhibitor, showing a cross-reactivity index of 14.6% (calculated from the molar concentration required to reduce the [³H]digoxin binding by 50%); the monodigotoxoside was even less reactive (cross-reactivity index 3.7%). The aglycone, digoxigenin, was an ineffective antigen for the new antibody (Figure 9a), and neither deslanoside nor the free digoxose sugar competes for digoxin binding (Figure 9b). Digitoxin and dihydroidigoxin showed a cross-reactivity index of 11% and 67%, respectively (Figure 9b).

We then compared the present antiserum, in the [³H]digoxin RIA, with a commercially available anti-digoxin antiserum and fluorescence polarization immunoassay, to measure the "digoxin" concentration of solutions containing known amounts of the glycosidic metabolites (diluted from a stock solution prepared by weighing) and to measure digoxin in the presence of equal concentrations of each of the glycosidic metabolites (Table 3). Only our present carbohydrate-specific anti-digoxin antibody was able to distinguish the metabolites from native digoxin.

**Discussion**

The narrow range for therapeutic as opposed to toxic doses makes digoxin one of the more difficult drugs to administer (19), particularly in infants (9, 21). The problem is compounded by considerable interindividual differences in digoxin metabolism (3) and absorption (22) in individuals with

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**Fig. 8. Measurement of anti-digoxin activity by ELISA**

The binding of anti-digoxin antibodies to a BSA–digoxin conjugate prepared by (A) reductive ozonolysis or (B) periodate oxidation of digoxin was determined as described in Methods. A, immune serum vs digoxin–BSA conjugate; O, immune serum vs BSA; •, nonimmune serum vs digoxin–BSA; □, nonimmune serum vs BSA. Bars indicate SD of determinations.

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**Fig. 9. Specificity of anti-digoxin serum**

Inhibition of the binding of [³H]digoxin by an aliquot of the immune serum was measured as described in Methods. A: digoxin, ∆: digoxigenin bidadigoxide; ⌧: digoxigenin monodigoxide; O: digoxigenin; B: digoxin, ▴: digitoxin, □: digitoxose; ○: dihydroidigoxin, ◇: deslanoside, □: nonimmune serum. Bars indicate range of duplicate determinations.
normal or impaired renal function (4, 9). These factors necessitate an accurate means for monitoring concentrations of digoxin in serum. In current clinical practice, various immunosassay procedures are used, based on the binding of a specific anti-digoxin antibody. However, the anti-digoxin antibodies in current use cannot distinguish between digoxin and its metabolites: bisdigiAoxide, monodiAoxide, and the aglycone, digoxigenin. Indeed, with the variations from antibody to antibody, these metabolites are sometimes more potent antigens for the anti-digoxin antibodies than is native digoxin (8).

We have verified this observation by using a standard RIA and commercially available antiserum (Table 1). Not only were the metabolites of digoxin in a standard sample detected, they were overestimated by as much as fourfold. This value was considerably reduced if the anti-digoxin antibodies were purified by affinity chromatography, such that digoxin and its metabolites are now equally potent antigens. The method we used to produce the digoxin affinity matrix (16) does not involve modification of the carbohydrate of digoxin. Nevertheless, even the affinity-purified antibodies cannot distinguish digoxin from its glycosidic metabolites. Because such discrimination might be possible with an antibody that recognizes the carbohydrate region of digoxin, we set out to synthesize a BSA–digoxin conjugate in which the carbohydrate moiety remains intact.

Reductive ozonolysis should provide an approach to this problem. As discussed above, we reasoned that, while the reactive intermediate (compound C, Figure 2) might be unstable owing to the proximity of the aldehyde and ester functions, formation and reduction of a Schiff's base at C-23 would stabilize the ester linkage. Thus, if an amine could be added directly during the reduction step, a stable conjugate might be formed. We used methylated BSA because unlike BSA, it is soluble in certain organic solvents. At basic pH, the efficiency of coupling was high: >40 digoxin molecules coupled per molecule of BSA, with an overall yield of ~30% digoxin bound (Figure 6). Extensive dialysis removed the unbound material. The resulting conjugate, unlike mBSA, stained positive for carbohydrate (Figure 6, lane g). Analysis of a sample by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that a new high-Mr protein species had been formed (not shown).

We used this conjugate to immunize a rabbit to produce anti-digoxin antibodies, and monitored the immune response with the [3H]digoxin-binding RIA. Serum having maximum binding in this assay showed no digoxin binding in the RIA with [125I]-labeled digoxin (15), in which the carbohydrate moiety of the "digoxin" tracer is removed and replaced with [125I]-labeled tyrosine. Commercial anti-digoxin antibodies bound digoxin in either assay. We concluded from this that the new anti-digoxin antiserum was binding, at least in part, to the carbohydrate moiety of digoxin—a possibility supported by ELISA. Using as antigen the mBSA–digoxin conjugate we had prepared via ozonolysis, we obtained a high-titer antiserum with minimal activity against unconjugated BSA. However, when the antibody activity was measured in a BSA–digoxin conjugate prepared by periodate oxidation of the terminal sugar (10), reactivity was only marginally greater than that observed for BSA alone (Figure 5b), again suggesting that the antibody recognizes the carbohydrate chain.

The discrepancy of anti-digoxin titer measured by the [3H]digoxin RIA and the ELISA reflects the lower sensitivity of this RIA method. However, because of the simplicity of the RIA, we used it in the subsequent studies of antibody specificity, which showed that the new antibody selectively binds digoxin molecules having an intact carbohydrate chain. Sequential removal of the glucose units reduced the antigenic reactivity (Figure 5a). The absence of an additional glucose residue at the non-reducing carbohydrate terminus (in the compound deaIsanoside) also resulted in the loss of antigenic activity (Figure 5b). The free sugar, digitoxose, did not cross react (Figure 5b), which suggests a requirement for the steroid moiety. Indeed, the digoxin analog, digitoxin, in which the hydroxyl group at C-12 of the steroid is missing, showed considerably reduced reactivity, although the carbohydrate chain is unaltered (Figure 5b). This indicates that conjugation of digoxin at the C-23 position is sufficiently far from C-12 to permit immune recognition at this site. As expected, dihydrodigoxin, in which the steroid lactone ring is saturated, shows significant cross reactivity.

We compared the new antibody as used in the [3H]digoxin RIA, with antibody prepared by immunization with BSA coupled to periodate-oxidized digoxin, used in a fluorescence polarization immunosassay in a manner similar to that previously done with the [125I]-labeled digoxin RIA (Table 1). The results (Table 3) clearly show that the commercial antibody grossly overestimates the digoxin concentration in samples containing digoxin metabolites. In contrast, the new antibody described in this paper, in which the intact carbohydrate moiety of digoxin is important for immunoreactivity, measures the digoxin concentration accurately. The presence of all of the digoxin metabolites (including dihydrodigoxin) did not affect the accurate quantification of digoxin.

Note added in proof: Recently, antidiAoxin antibodies of similar specificity have reportedly been raised by using an immunogen prepared by BSA conjugation at the C-17 or C-12 position of digoxin (Shimada K, Minusawa S, Ohkubo T, Nambara T. Chem Pharm Bull 32:2301–2306, 1984).
References


