

Short Communication

Phenylboronic acid as a versatile derivatization agent for chromatography of ecdysteroids

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(First received November 8th, 1991; revised manuscript received January 21st, 1992)

ABSTRACT

Phenylboronic acid can react with the diol group to form a cyclic boronate. This reagent forms stable boronates with ecdysteroids possessing a 20,22-diol group in quantitative yield. The stability of these boronates enables the presence of a 20,22-diol group in ecdysteroids to be detected on the basis of their changed chromatographic properties. On the basis of this reaction, simple and efficient thin-layer and high-performance liquid chromatographic methods were developed.

INTRODUCTION

Ecdysteroids are significant hormones controlling the mechanism of moulting and metamorphosis of arthropods. They regulate whole series of their important physiological functions. The steroid character of these substances and their unusually broad distribution in living organisms (especially in plants) indicates that they may also have further, so far undiscovered, biological functions. The ecdysteroids from plants (phytoecdysones) [1] occur in a richer structural variety than zooecdysones [2]. They are usually more hydroxylated, either in the free form or in an ester or glycosidic form. They are nearly always present in complex mixtures of one or two main constituents and of several additional minor derivatives or analogues showing some small structural variations. The composition of the main and minor constituents is often changed in the course of phylogenetic development and, moreover, their content varies in the different organs of the

plant. It is therefore important to have suitable simple and specific methods for the rapid detection and facile identification or characterization of these substances in complex mixtures of plant extracts. One such method is based on the use of the rapid and quantitative reactivity of phenylboronic acid with the diol group in the ecdysteroid molecule [3].

The usefulness of boronic acids has been demonstrated in carbohydrate chemistry [4]. Methaneboronic acid [5], phenylboronic acid [6] and ferroceneboronic acid [7] have been used for the gas chromatographic–mass spectrometric analysis of bifunctional compounds. Diphenylborate–ethanolamine complex has been used for the isolation of catecholamines from body fluids [8]. Phenylboronic acid has been used for the protection of 1,2- and 1,3-diols [9] and in the organic synthesis of chiral compounds [10]. It produces cyclic boronates. The stability of the boronates depends on the nature of the diols used. Many boronates are moisture sensitive and unstable under protic conditions. More hin-

dered diols form more hydrolytic stable boronates. Different solvents can be used for the preparation of boronates. Their choice depends on the solubility of the reacted diol. Common solvents used for this reaction are pyridine, tetrahydrofuran, dimethylformamide (DMF) and acetone. Water is liberated in the course of the esterification reaction, hence molecular sieves or water scavengers such as 2,2-dimethoxypropane are usually added to the reaction mixture for the preparation of moisture-sensitive boronates.

With ecdysteroids, phenylboronic acid has been used for the protection of the diol system in the side-chain of cyasterone [11]. Other aromatic boronic acids, *e.g.*, phenanthreneboronic acid [12] and dansylaminophenylboronic acid [13], have been used as fluorescence labels in the analysis of ecdysteroids. Phenylboronic acid immobilized on silica gel has been used for the solid-phase extraction of ecdysteroids [14]. Ecdysones frequently contain two diol systems, one in the 2,3-position and the other in the 20,22-position of the side-chain. Phenylboronate

is formed exclusively from the diol in the side-chain (Fig. 1), even if an excess of reagent is present in the reaction mixture. The reaction is quantitative and ecdysteroid boronates are unusually stable under protic conditions. The derivatization reaction results in a change in the chromatographic behaviour of reacted compounds. This property can be utilized for simple checking of the presence of the 20,22-diol system in the ecdysteroid molecule based on chromatographic methods.

EXPERIMENTAL

Chemicals

Dichloromethane and methanol (Lachema, Brno, Czechoslovakia) were redistilled and water was deionized and redistilled. Ecdysteroids, *i.e.*, 20-hydroxyecdysone-2,3-acetonide (**I**), ponasterone A (**II**), 20-hydroxyecdysone-20,22-acetonide (**III**), polypodine B (**IV**), ecdysone (**V**) and 20-hydroxyecdysone (**VI**), were isolated from *Blechnum spicant* L. [15].

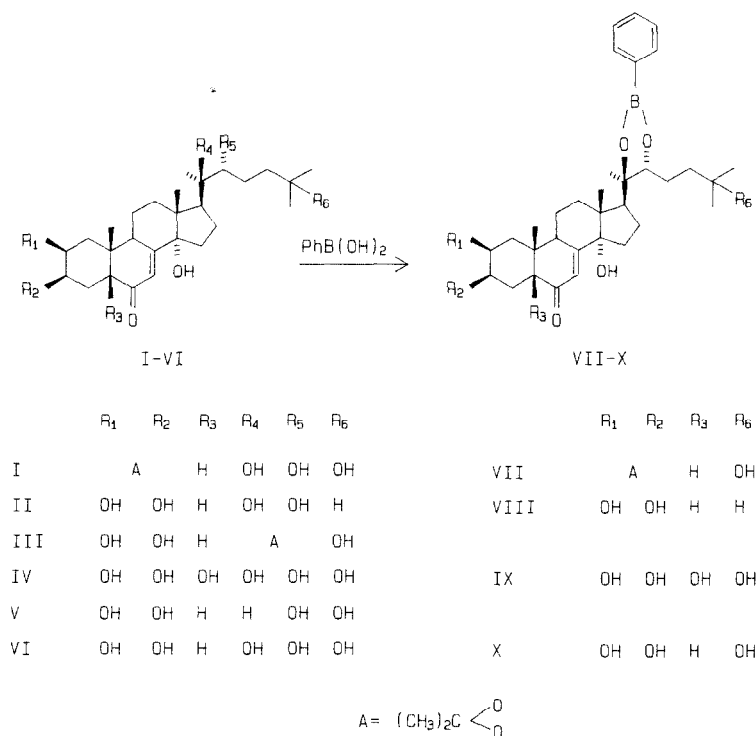


Fig. 1. Structures of ecdysteroids and their phenylboronate derivatives.

Phenylboronic acid (phenyldihydroxyborane, benzenboronic acid) was prepared according to a common procedure [16].

Preparation of reference compounds, boronates VII-X

All boronates were prepared according to the following general procedure. Ecdysteroid (3–8 mg) was dissolved or suspended in a suitable solvent (methanol, DMF or acetone). Phenylboronic acid (1.2 equiv.) was added and the reaction mixture was stirred for 5–10 min or, when acetone was used, up to dissolution and then for an additional 5 min. After evaporation of the solvent, the reaction mixture was subjected to normal-phase high-performance liquid chromatography (HPLC), giving pure boronate in 80–90% yield. Identification of the reference boronates VII–X was accomplished by IR, mass and ^1H NMR spectroscopy [17].

Chromatography

Thin-layer chromatography (TLC) was performed in a glass chromatography tank containing an appropriate solvent. The atmosphere in the tank was allowed to presaturate before performing chromatographic separations. Merck silica gel 60 HPTLC aluminium sheets and Merck silica gel RP-8 reversed-phase HPTLC plates were used. The silica gel sheets were developed in methanol–chloroform (15:85, v/v) and the reversed-phase plates in methanol–water (70:30, v/v). The developed chromatograms were revealed by the induced fluorescence under UV light after spraying with sulphuric acid [18].

HPLC experiments were performed on a liquid chromatograph consisting of two Knauer Model 64 HPLC pumps and a Knauer variable-wavelength monitor. Samples were injected through a Rheodyne Model 7125 sampling valve. A Separon SGX (7 μm) silica gel column (250 mm \times 4 mm I.D.) was used with dichloromethane–methanol–water (88:11:1, v/v/v) for isocratic elution at a flow-rate of 1.5 ml/min.

Procedure for TLC

Ecdysteroids dissolved in methanol were applied to the TLC plate, each compound as two spots. After evaporation of the solvent from the sample spots, an acetone solution of phenylboronic acid

was applied to the second sample spot of each ecdysteroid. After 5 min the TLC plates were developed with a suitable solvent.

Procedure for HPLC

A solution of ecdysteroids I–VI in the mobile phase was used for the derivatization reaction. A dichloromethane solution of phenylboronic acid (up to 10 equiv.) was added to the solution of ecdysteroids containing ca. 15 μg of each compound. After shaking, the reaction mixture was allowed to react for 5 min and was then injected into the HPLC system.

Methanol and methanol–water (3:1, 1:1, 1:3 and 1:9, v/v) solutions of a crude extract containing 20-hydroxyecdysone (ca. 11%) from roots of *Leuzea carthamoides* (Willd.) were treated with a methanolic solution of phenylboronic acid (20 equiv. based on 20-hydroxyecdysone content). The reaction mixture was allowed to react for 15 min and was then injected into the HPLC system.

RESULTS AND DISCUSSION

TLC analysis

The reactivity of the side-chain diol-containing ecdysteroids with the phenylboronic acid on the sorbent surface was sufficiently fast and quantitative to provide unambiguous results. R_F values from silica gel plates are summarized in Table I. If a diol is present in the side-chain (compounds I, II, IV and VI), a difference between the R_F value of the original ecdysteroid and that of the ecdysteroid after reac-

TABLE I
 R_F VALUES OF ECDYSTEROIDS AND THEIR BORONATES ON A SILICA GEL PLATE DEVELOPED WITH METHANOL–CHLOROFORM (15:85, v/v) AND DETECTED BY SPRAYING WITH SULPHURIC ACID

Ecdysteroid	R_F	Compound after reaction	R_F
I	0.51	VII	0.72
II	0.40	VIII	0.64
III	0.51	III	0.51
IV	0.27	IX	0.49
V	0.28	V	0.28
VI	0.22	X	0.42

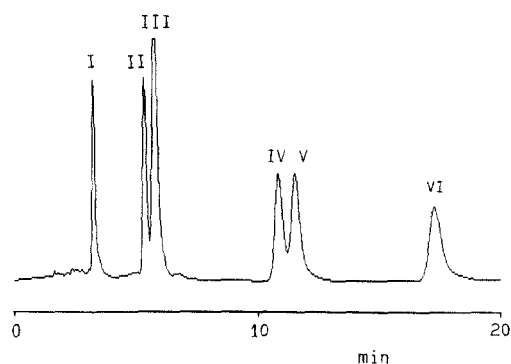


Fig. 2. Separation of ecdysteroids I-VI by silica HPLC.

tion was observed. With the reversed-phase RP-8 plates, boronates exhibit a shift of spots in comparison with the parent ecdysteroids. However, the shape of the boronate spots exhibited considerable deformation.

HPLC analysis

The HPLC separation of six ecdysteroids on a silica gel column is demonstrated on Fig. 2. Four of them, viz., I, II, IV and VI, contain a diol in the side-chain and hence they are able to react with phenylboronic acid. The results of this small-scale reaction are demonstrated in Fig. 3. The presence of the diol function can be recognized from the total disappearance of the original ecdysteroid peaks. New peaks related to the boronates formed appear at shorter retention times. The relative elution order

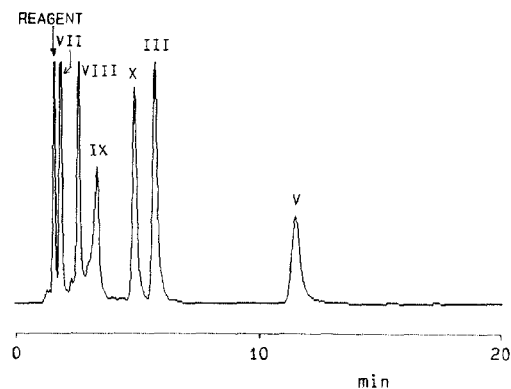


Fig. 3. Separation of ecdysteroids after reaction with phenylboronic acid by silica HPLC. Unreactive ecdysteroids, III and V; boronates formed, VII-X.

of the boronates is the same as that of the original ecdysteroids. The derivatization reaction is very rapid and in principle it could be carried out directly in the sample loop. Its quantitative course is limited here, however, by the reduced possibility of mixing the reaction components.

All efforts to perform such HPLC analyses in the reversed-phase mode failed, probably owing to the limited stability of the prepared boronates under these conditions. For *Leuzea carthamoides* extract the results were satisfactory when methanol was used because in this solvent reaction was complete. Equilibrium between free 20-hydroxyecdysone and its boronate occurred in water-containing solutions. The boronate-to-ecdysteroid ratio depends on the water content in reaction mixture. In methanol-water solution (10:90, v/v) only a 30% yield of the boronate was achieved.

In both TLC and HPLC analyses, the use of the normal-phase mode was more advantageous. The results of TLC analysis were equally conclusive as those of HPLC analysis, but the TLC method is less time consuming. Both methods have several advantages. They are fairly rapid and simple and they afford important structural information from microgram amounts of material. The information can be obtained directly by analysing complex mixtures, without isolation of the pure compounds.

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