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MONOTERPENE BIOSYNTHESIS IN THE LIVERWORT *CONOCEPHALUM CONICUM*: DEMONSTRATION OF SABINENE SYNTHASE AND BORNYL DIPHOSPHATE SYNTHASE

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Conocephalum conicum*; Hepaticae; liverwort; biosynthesis; sabinene; bornyl acetate; geranyl diphosphate; monoterpene cyclase; sabinene synthase; bornyl diphosphate synthase.

Abstract—(−)-Sabinene is the major monoterpene produced by a European strain of the liverwort *Conocephalum conicum*. A cell-free extract from *in vitro* cultured plants catalysed the cyclization of geranyl diphosphate to sabinene. The responsible monoterpene cyclase was partially purified and characterized as an operationally soluble enzyme of M_r 65 000, with a pH optimum at 7.5 and a requirement for a divalent metal ion as the only cofactor, with Mg^{2+} preferred. The general properties of the sabinene synthase from *C. conicum* resemble those of other monoterpene cyclases from gymnosperms and angiosperms. A North American strain of the liverwort produces (+)-bornyl acetate as the major monoterpene and it was demonstrated that bornane-type monoterpenes are derived from geranyl diphosphate in this liverwort, as in higher plants, by the action of bornyl diphosphate synthase.

INTRODUCTION

Approximately 70 monoterpenes, 480 sesquiterpenes and 220 diterpenes, many with unique carbon skeletons, have been isolated from liverworts in which these lipophilic metabolites are deposited in specialized intracellular oil bodies [1–5]. A number of liverwort terpenoids exhibit interesting organoleptic, pharmacological and other biological activities [1–5]. In spite of the remarkable diversity of terpenoids produced by liverworts, investigations directed to the biosynthesis of these compounds in nonvascular plants are rare [6] compared to the numerous studies on terpenoid metabolism in higher plants [7–9].

As an experimental system for comparative biochemical investigations, two varieties of the liverwort *Conocephalum conicum* were chosen, one producing (−)-sabinene (**2**), the other (+)-bornyl acetate (**5**), as the major volatile monoterpene components (see Scheme 1) [10]. The enzymatic cyclizations of the universal precursor geranyl diphosphate (**1**) leading to these terpenoid metabolites in higher plants have been well defined, thus providing the opportunity for comparing monoterpene cyclases from phylogenetically very different plants which might provide insights on the evolutionary origins and structure–function relationships of these important catalysts. Additionally, the use of cultured liverworts, as described here, offers both independence from native collection and abundant supply of homogenous plant material of reproducible composition. In this paper, we report on the isolation and comparative properties of sabinene synthase from *C. conicum* and describe the bornyl diphosphate synthase from this liverwort, thus demonstrating that bornane-type monoterpenes are also derived by the same, unusual cyclization mechanism utilized by higher plants [11].

RESULTS AND DISCUSSION

Variation in the monoterpene content of *C. conicum* of different geographic origin is well documented [10]. An acquisition from France produces mainly (−)-sabinene (**2**), whereas *C. conicum* of Japanese origin produces (+)-bornyl acetate (**5**) as the major monoterpene with substantial amounts of (−)-sabinene [12–14]. The steam distilled essential oil of a German

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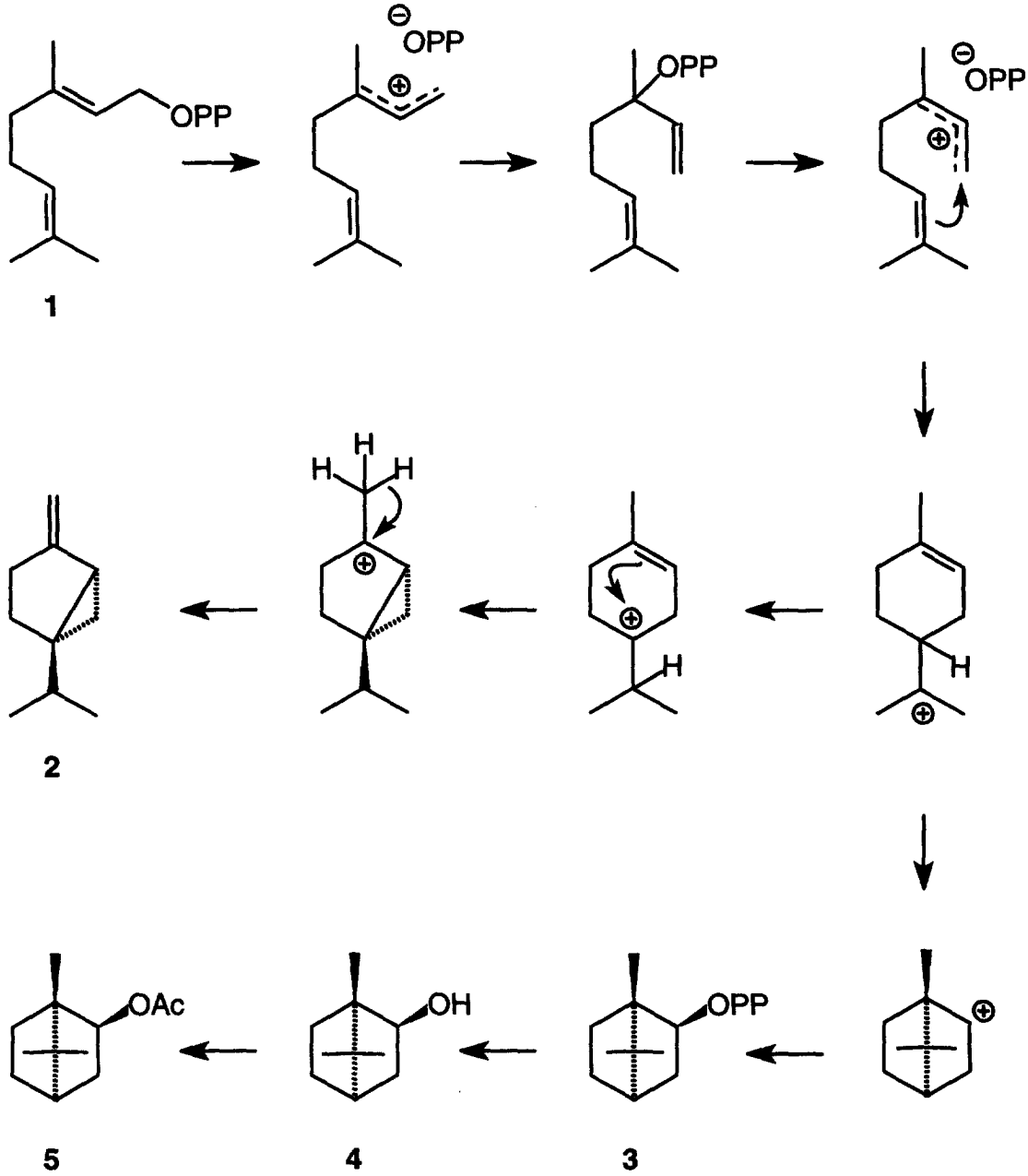

Scheme 1. Cyclization of geranyl diphosphate (**1**) to (−)-sabinene (**2**) and (+)-bornyl diphosphate (**3**). *OPP* denotes the diphosphate moiety, *Ac* the acetyl group.

strain of *C. conicum* used in this study was shown by GLC-MS analysis to contain sabinene [presumably the (−)-enantiomer (**2**)] as the major component (55%), with very low levels of bornyl acetate (0.1%) and borneol (0.1%); the remainder of the oil appeared to be composed primarily of sesquiterpenes and acyl lipids. A North American variety of *C. conicum* was shown by similar analysis to contain bornyl acetate [presumably the (+)-enantiomer (**5**)] as the major component of the distilled oil (48%), with lesser amounts of borneol (**4**) (2.2%), traces of sabinene (0.2%), and a similar spectrum of other metabolites.

Enzyme extracts were prepared from quick-frozen gametophytes of both liverwort strains by methods previously developed for the isolation of other terpenoid synthases that minimize the deleterious effects of co-extracted resinous and phenolic materials [15]. Incubation of these preparations with 5μM [1-^{3}H]geranyl diphosphate (**1**) and 10 mM $MgCl_2$ gave rise to very different distributions of cyclic monoterpene products depending on the culture line used. The homogenate of the strain that produced primarily sabinene yielded a monoterpene olefin as the only product, and this metabolite was identified as sabinene

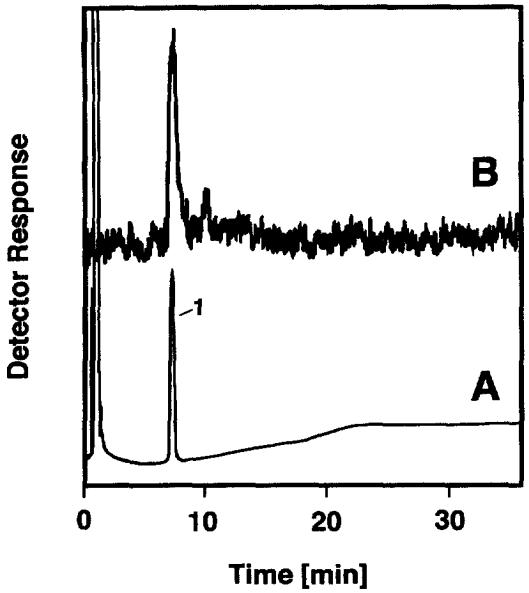

Fig. 1. Radio-GLC separation of the monoterpene olefins generated from [1-^{3}H]geranyl diphosphate by a preparation from the variety of *C. conicum* that produces sabinene. Tracing A is the thermal conductivity detector response to the authentic sabinene standard. Tracing B is the radioactivity detector response to the biosynthetic olefin products. Conditions of the analysis are described in the text.

(**2**) based on radio-GLC coincidence with the authentic standard (Fig. 1). Boiled control preparations evidenced negligible activity for the production of sabinene.

Centrifugal fractionation of the homogenate indicated that the sabinene synthase was operationally soluble (negligible activity was associated with particulate fractions), and the enzyme was partially purified by anion-exchange chromatography on *O*-diethylaminoethyl cellulose. The partially purified enzyme showed the same product profile derived from geranyl diphosphate as the crude extract. A native M_r of 65 000 was determined for the sabinene synthase by gel permeation chromatography; this size is similar to that of sabinene synthase from *Salvia officinalis* [16] and to other monoterpene synthase from higher plants [7, 9].

Following preliminary evaluation of assay conditions, the pH optimum and optimum buffer for sabinene synthase activity were determined (pH 7.5 HEPES, with 80% activity at pH 7.8 and 8.0). The enzyme evidenced the greatest stability at pH 7.0. All terpenoid synthases require a divalent metal ion for activity [11, 17, 18] which is thought to bind to the anionic diphosphate group of the prenyl substrate and assist in the ionization step of the reaction [19, 20]. In the absence of divalent metal ion, the sabinene synthase was completely inactive. Activity was fully restored by the addition of 10 mM Mg^{2+}. Substitution of Fe^{2+} (10 mM) showed 90% of the activity obtained with 10 mM Mg^{2+}. Other divalent cations were less effective in supporting catalysis ($Mg^{2+} > Fe^{2+} > Mn^{2+} \gg Zn^{2+} > Co^{2+} > Ni^{2+} > Cu^{2+}$; compared at 10 mM as the chloride salts). Interestingly, in cation requirement (efficient utilization of Mg^{2+} and Fe^{2+}), the sabinene synthase of *C. conicum* resembles more closely the monoterpene synthases of gymnosperms than those from angiosperms [21]. A K_m value of 4.0 μM for geranyl diphosphate was determined for sabinene synthase by computer assisted Lineweaver–Burk plotting; this K_m value is typical of the enzyme class [7, 11]. Thus, in general properties, the sabinene synthase from *C. conicum* resembles other monoterpene synthases from higher plants [7, 11, 21, 22] and is very similar to the limonene synthase of *Ricciocarpos natans*, the only other monoterpene synthase from a liverwort to be isolated and described [6]. However, the limonene synthase of *R. natans* has a molecular weight of 51 000 [6], whereas the sabinene synthase of *C. conicum* has an M_r of approximately 65 000.

When the cell-free extract of the *C. conicum* strain that produced principally (+)-bornyl acetate was assayed in the presence of [1-^{3}H]geranyl diphosphate and $MgCl_2$ as before, no monoterpene olefins were formed. However, the organic extract of the reaction mixture did contain both borneol and geraniol as determined by radio-GLC analysis. The latter monoterpenol arises from hydrolysis of the substrate by endogenous phosphatases present in the preparation, but the origin of borneol by direct cyclization of geranyl diphosphate or by hydrolysis of bornyl diphosphate formed upon cyclization of the geranyl substrate could not be readily determined by separation of the contaminating phosphatases from the cyclase enzyme because too little activity was available in these tissue extracts. To determine if bornyl diphosphate was formed as the reaction product, the assay was modified to search specifically for this metabolite [23]. Thus, following the incubation, the assay mixture was treated with acid to solvolyse the residual substrate to a mixture of acyclic monoterpenols. These and any other lipophilic products (including any borneol) were then extracted with hexane and the remaining aqueous phase was treated with phosphatase and apyrase to enzymatically hydrolyse any bornyl diphosphate present. Hexane extraction yielded a radiolabeled product that was liberated by this treatment and that was separated by TLC and shown to be coincident with authentic (+)-borneol. The product was confirmed as borneol based on radio-GLC coincidence with the authentic standard (Fig. 2). Thus, the original enzymatic product present was bornyl diphosphate, indicating that bornane-type monoterpenes in this liverwort are formed by the same route as in *Salvia officinalis* and *Tanacetum vulgare* [23, 24], and suggesting that bornyl acetate is formed in *C. conicum* by hydrolysis of the initial diphosphate ester formed to yield borneol which is then acetylated (Scheme 1). These results, and earlier work with limonene synthase from *R. natans* [6], suggest that both the reaction

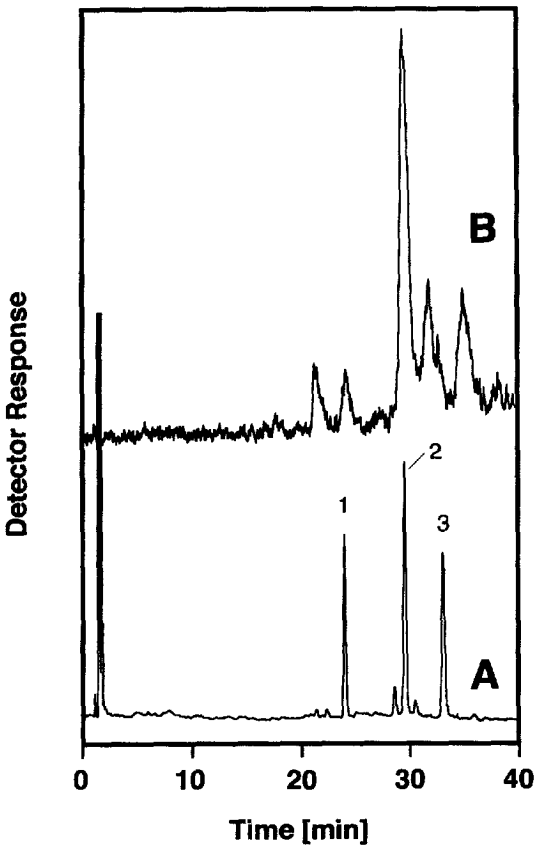

Fig. 2. Radio-GLC separation of the monoterpenol fraction generated from [1-^{3}H]geranyl diphosphate (after hydrolysis) by a preparation from the variety of *C. conicum* that produces (+)-bornyl acetate. Tracing A is the thermal conductivity detector response to authentic standards of (±)-linalool (**1**), (+)-borneol (**2**) and geraniol (**3**). Tracing B is the radioactivity detector response to the biosynthetic monoterpenol products. One of the minor radioactive components corresponds to linalool derived by solvolysis of the labeled substrate; the other minor radioactive components are presently unassigned. Conditions of the analysis are described in the text.

mechanisms and enzymes of monoterpene biosynthesis in liverworts are very similar to those of higher plants. The evolutionary significance of this observation must await the cloning and sequencing of the corresponding liverwort monoterpene synthase genes for comparison to their counterparts from higher plants.

EXPERIMENTAL

Plant materials, substrate and reagents. The sterile culture of *C. conicum* was initiated from surface disinfected gametophytes [25] obtained from a native site near Heidelberg, Germany. The culture was grown in 250 ml Erlenmeyer flasks under continuous light (1000 Lux fluorescent lamps, photon flux density 15 μmol photons·m^{-2}·s^{-1} (400–700 nm)) at 20° on Gamborg's B5 medium without phytohormones [26] containing 2% sucrose solidified with 0.9% agar. Plant material was harvested after 6 weeks of growth. Plant material from North America was collected at a native site near Clarkia, Idaho, U.S.A. and was similarly maintained in sealed, light transparent containers under continuous light as above at 20°. Fresh three-week-old thallus tips were used for the biosynthetic studies. The essential oil of each liverwort was obtained by steam distillation of the tissue and was analysed by combined capillary GLC-MS using a protocol described previously [27].

The preparation of [1-^{3}H]geranyl diphosphate (250 Ci·mol^{-1}) has been described [28], and the monoterpene standards were from our own collection. All other biochemicals and reagents were purchased from Sigma Chemical Co. or Aldrich Chemical Co., unless otherwise noted.

Enzyme isolation and purification. Approximately 50 g (fresh wt.) of tissue was frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. The frozen powder was immediately slurried with cold extraction buffer (150 ml, 20 mM HEPES buffer (pH 7.0; 2 ml g^{-1} fr. tissue wt.) containing 10 mM sodium ascorbate, 10 mM β-mercaptoethanol, 10 mM $Na_2S_2O_5$, 50 mM sorbitol, 10% glycerol, 1% PVP ($M_r \approx 10\,000$), and 0.3 g g^{-1} fr. tissue wt. each of PVPP and polystyrene resin (Amberlite XAD-4)). After stirring the suspension for 30 min, and centrifugation at 24 000 *g* for 30 min and at 105 000 *g* for 90 min, the soluble supernatant and pelleted membranous fractions were assayed for monoterpene synthase activity. The pellets, which were devoid of synthase activity, were discarded and the supernatant, which contained the monoterpene synthase activity, was used as the enzyme source. Centrifugation and all subsequent steps were carried out at 0–4°.

For routine purification, the 24 000 *g* supernatant from above was added to 30 g of *O*-diethylaminoethylcellulose (Whatman DE-52) that had been extensively washed with extraction buffer containing 1 M KCl and then equilibrated with extraction buffer (without KCl). The suspension was stirred for 20 min before centrifugation at 1000 *g*. The supernatant was discarded and the pelleted matrix was poured into a column (25 mm i.d. × 200 mm) and rinsed with 100 ml of extraction buffer, then with 100 ml of extraction buffer containing 50 mM KCl. The sabinene synthase or bornyl diphosphate synthase activity was subsequently eluted with extraction buffer containing 100 mM KCl. Fractions (10 ml) containing the appropriate monoterpene synthase were pooled (≈150 ml) and concd by ultrafiltration (nominal membrane cut-off of 30 000) in a stirred cell to a final volume of 20 ml.

Enzyme assay and product identification. A series of optimization studies led to the development of a standard assay procedure in which 50 μl of the enzyme preparation (10–100 μg protein; protein concn estimated by the Bradford method [29] using the Bio-Rad

reagent with lysozyme as standard) was diluted to 200 μl with extraction buffer containing 10 mM $MgCl_2$ in a 1.7 ml Eppendorf tube. When it was necessary to modify these conditions, for example to determine the response to pH or to different cations, the samples were dialysed to the appropriate conditions. The reaction was initiated by the addition of 5 μM [1-^{3}H]geranyl diphosphate (0.25 μCi) followed by gentle mixing and incubation for 1 hr at 32°. After incubation, 1 ml hexane was added and the reaction mixture was vigorously mixed and, following centrifugation to separate phases, 0.9 ml of the hexane layer was transferred to a new Eppendorf tube containing 100 mg silica gel [SilicAR 60A (Millinckrodt)], followed by mixing and centrifugation to pellet the matrix and afford the hydrocarbon fraction, free of oxygenated metabolites. Ether extraction of the silica gel (1 ml Et_2O) provided the oxygenated monoterpenes. A 0.5 ml portion of the monoterpene hydrocarbon fraction and of the monoterpene alcohol fraction was analysed by liquid scintillation counting in 10 ml of a cocktail consisting of 0.4% Omnifluor (DuPont/New England Nuclear) dissolved in 30% EtOH in toluene (^{3}H efficiency, $\approx$42%). The assay at 5 μM substrate was linear with respect to protein concn up to at least 20 μg ml^{-1} for a 1 hr incubation period, and all assays were conducted under these linear conditions. Boiled controls evidenced negligible activity with [1-^{3}H]geranyl diphosphate as substrate.

The identity and purity of the biosynthetic products were determined by radio-GLC [30]. To prepare sufficient product, the assay was scaled-up using 1 ml of enzyme prepn and the incubation time was extended to 3 hr. The monoterpene hydrocarbon fraction, obtained after chromatography of the hexane extract over silica gel, was diluted with authentic standards, concd and analysed. The conditions for analysis on the 3 mm o.d. × 4 m AT1000 column (15% polyethylene glycol ester on Gas-Chrom Q) were 100° isothermal (5 min) then programmed to a final temp. of 200° at 5° min^{-1}, with helium (34 ml min^{-1}) as carrier, injector at 230°, and thermal conductivity detector at 250° with current of 150 mA. The identity of the enzymatically-generated monoterpene olefin ((−)-sabinene) was verified by comparison of the R_t to the authentic standard. In the assay for bornyl diphosphate, the bulk of the [1-^{3}H]geranyl diphosphate remaining after enzyme incubation was removed by addition of 100 μl of 0.1 M HCl to solvolyse the allylic substrate (primarily to linalool, geraniol and nerol), followed by hexane extraction. The pH of the residual aqueous layer was adjusted to 6.5 with dilute NaOH, and the mixt. was incubated with 1 unit each of apyrase and acid phosphatase to hydrolyse phosphate esters of borneol. Following this enzymatic hydrolysis step and hexane extraction, the extract was diluted with authentic (+)-borneol, concd and purified by TLC (silica gel, hexane–EtOAc, 7:3). The band corresponding to borneol ($R_f \approx 0.49$) was removed, the silica gel was extracted with Et_2O, and the extract was concd for radio-GLC as before (the column was programmed from 150° (5 min isothermal) to 230° at 5° min^{-1}).

Enzyme characterization. To determine the optimum pH and buffer type for monoterpene synthase activity, assays were conducted at half pH unit intervals in Tris (pH 6.5–8.5), HEPES (pH 6.5–8.5), phosphate (pH 7.0 and 7.5), Bis-Tris propane (pH 7.5–10.0) and AMPSO (pH 7.0–8.5), all at 30 mM containing 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 10% glycerol. For studies of divalent cation requirement, preparations were desalted into 20 mM HEPES buffer (pH 7.0) containing 10 mM β-mercaptoethanol, 10 mM sodium ascorbate, 10 mM $Na_2S_2O_5$ and 10% glycerol by passage through an Econo-Pac 10 DG column (Bio-Rad).

To estimate the native molecular mass of the sabinene synthase, the partially purified, concd enzyme prepn was subjected to size-exclusion chromatography on a calibrated Sephacryl S-200 column (1.6 cm × 90 cm, Pharmacia), developed with extraction buffer at pH 7.0 containing 100 mM KCl at a flow-rate of 0.5 ml min^{-1}. The K_m value for geranyl diphosphate with the sabinene synthase was determined by computer assisted Lineweaver-Burk plotting of the reaction rates obtained over a substrate concentration range of 0.5 to 15 μM.

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