DEREPLICATION OF PHORBOL BIOACTIVES: *LYNGBYA MAJUSCULA* AND *CROTON CUNEATUS*

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ABSTRACT.—*Lyngbya majuscula* and *Croton cuneatus* were used as prototypes for the dereplication of phorbol ester receptor binding activity using a combination of hplc-uv and online phorbol dibutyrate (PDBu) receptor binding and batch fractionation over either Si gel or diol-bonded Si gel. Debromoaplysiatoxin was responsible for the bioactivity of *Lyngbya*, whereas a complex of potent phorbol esters was detected in *C. cuneatus*.

The use of a simple phorbol dibutyrate (PDBu) receptor binding assay in our laboratory to examine terrestrial and marine plant extracts has identified a large number of bioactive organisms (1). A variety of structural types from these sources are known to interact with the PDBu receptor, including phorbol diterpenes, bryostatins, teleocidins, and aplysiatoxins (2). The large number of PDBu-active extracts made it desirable to identify the samples containing known active compounds or compound classes in a rapid fashion, without investing time in traditional bioassay-guided fractionation and full structure elucidation. This process of quickly identifying known chemotypes is termed dereplication within the antibiotic field. We present here examples of how the PDBu assay combined with hplc-uv can be used to rapidly dereplicate active compounds. The use of this method is illustrated for one marine source [*Lyngbya majuscula* (Dillwyn) Harvey (Oscillatoriaceae)] and one terrestrial source [*Croton cuneatus* Klotzch (Euphorbiaceae)]. Use of the method in dereplication problems is discussed.

The first example, the filamentous blue-green alga *L. majuscula*, has been reported to contain either phenolic macrolides such as debromoaplysiatoxin or indoles such as lyngbyatoxin, depending on the collection site and microbial associates present (3). Both classes of compounds are known to be active in the phorbol receptor system (2). A number of other sea grasses and macroalgae from the same collection site as the active *Lyngbya* were also PDBu bioactive; our goal was to identify the chemotype present in the *Lyngbya* and to determine if *Lyngbya* contamination was responsible for the bioactivity in the other samples from the same site.

The second example, *C. cuneatus*, was initially field-identified as a species of *Alchornea*. Because the genus *Alchornea* is placed in subfamily Acalypheae in Webster's treatment of the Euphorbiaceae (4) and we have found most of the phorbol ester bioactivity in two other subfamilies (1), our goal was to determine if the activity we observed was due to phorbol esters or to some other novel chemotype. A critical reexamination of botanical vouchers later showed this plant to be *C. cuneatus*.

These dereplication problems were addressed by rapid but gentle prefractionation steps and a combination of hplc with uv diode array detection, with microfractions directly examined in a PDBu competition binding assay. We have applied similar methods to analysis of bryostatin in *Bugula neritina* (5).

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES.—Analytical hplc was performed with a Perkin-Elmer

Series 410 quaternary pump equipped with an ISS-100 autosampler. Analytical columns were Rainin Dynamax cartridges (3 μ, 60Å, 4.6 mm $\times$ 10 cm C_{18}). Detection was done with a Waters 990 diode array system, and uv spectra were acquired using this system as well. Preparative hplc was done with a Milton Roy CM 4000 pump and SM 4000 variable uv-vis detector. Preparative columns were Rainin Dynamax cartridges (8 μ, 21.2 mm and 41.4 mm $\times$ 25 cm). Analytical chromatography, except where noted, was done with a gradient starting at 50% MeCN in H_2O for 2 min, followed by a linear gradient to 100% MeCN over 7 min, then 100% MeCN. Preparative gradients used the same parameters with gradient segments twice as long as for analytical runs. Ms was performed on a VG 70-250 in positive ion fab mode from a matrix of glycerol/DMF. Gc-ms was done on a DB-1 capillary column (0.32 mm $\times$ 30 m), 25 μ film coating, He carrier gas, on column injection with a temperature program from 105° at 20°/min to 200°, then 8°/min to 300°. Mass spectral parameters (VG 70–250) were ci mode NH_3, 1 Torr, 70 eV, resolving power 1000, 100 μA trap current, source temperature 180°, scanning from 650 to 40 m/z at 2 sec/decade.

PLANT SAMPLES.—*L. majuscula* and the other specimens from collection site 1 were collected by Ernani G. Menez near Chanaryan, Batan Island, Batanes, Philippines (20°26'20" N, 121°57'20" E) on a reef flat in a shallow bay area, rocky bottom with sand, April 30, 1986. *L. majuscula* from site 10 was collected near Apo Island, Negros Oriental, Central Visayas, Philippines (09°04'10" N, 123°16'00" E), rocky promontory and coral rubble area with sandy bottom, on June 6, 1986. Vouchers are in the Smithsonian Institution. *C. cuneatus* (Daly 5095) was collected by Douglas C. Daly in floodplain forest, Depto. Loreto, Prov. Requena, Dtto. Sapuena, Rio Ucayali, Supay Cocha, Cano Supay, Peru on April 16, 1987, local name "hipururu rojo." The bark was reported to be placed in cane liquor, aged, and drunk for rheumatism. The bark and leaves were collected separately. Voucher specimens are located in the New York Botanical Garden. The *Croton* plant samples were air-dried in the field and then processed by the method reported by McCloud *et al.* (6). Marine plant samples were frozen on collection, lyophilized, and then processed by the same method.

PHORBOL DIBUTYRATE BINDING ASSAY.—The PDBu binding assay was carried out as reported in Beutler *et al.* (1) and De Vries *et al.* (7).

ON-LINE PDBu BINDING.—Chromatographic fractions were deposited directly in tubes using a fraction collector (ISCO Foxy). If the volume of MeCN was less than 50μl, the tubes were used directly to perform the binding assay. For larger volumes the solvent was evaporated by placing the tubes in a vacuum oven at room temperature for several hours.

FRACTIONATION OF *L. MAJUSCULA* FROM STATION 1.—The CH_2Cl_2/MeOH extract of this collection had a PDBu IC_{50} of 0.29 μg/ml, while the collection of *L. majuscula* from station 10 was inactive at 100 μg/ml. The active crude extract was examined directly by analytical hplc [MeCN-H_2O (50:50), gradient to 100% MeCN]. A major uv-absorbing peak in the chromatogram was found to match standard debromoaplysiatoxin in both retention time and uv spectrum. On-line binding of this chromatogram with 10 μg of crude CH_2Cl_2/MeOH extract injected showed peaks of activity corresponding to peaks 5 and 7 (Figure 1), peak 5 correlated with the debromoaplysiatoxin standard. The active crude extract (55 mg) was coated on dicalite (1:10), slurried over an equal volume of flash silica, and batch-eluted with four to five column volumes of hexane, toluene, CH_2Cl_2, $CHCl_3$, EtOAc, Me_2CO, and MeOH in sequence. Bioassay with PDBu revealed that the EtOAc fraction had an IC_{50} of about 1 μg/ml and all other fractions were inactive at that level; recovery of bioactivity was only 4%. Because the debromoaplysiatoxin group of compounds is known to be sensitive to acidic chromatography conditions (3) (e.g., silica, MeCN, $CHCl_3$), we next used diol-bonded phase material and avoided acidic solvents. Crude extract (51 mg) was coated on 510 mg of diol media (Sepralyte, Analytichem, 40 μ particle size). The coated media was slurry packed over an equal volume of uncoated diol media and batch eluted with 30–40 ml each of hexane, CH_2Cl_2, EtOAc, Me_2CO, and MeOH. In this case the EtOAc eluate (7.1 mg, IC_{50} <20 ng/ml) contained >95% of the PDBu activity, and the Me_2CO eluate the remainder, with quantitative recovery of bioactivity. The mass distribution between fractions was comparable to the silica column, and total mass recoveries were ca. 85% in both cases.

FRACTIONATION OF *C. CUNEATUS*.—The leaf extract had an IC_{50} of 0.74 μg/ml vs. 3.7 μg/ml for the bark extract from the same collection; therefore, the leaf extract was fractionated. A conventional partition-based scheme was not successful in maintaining bioactivity. The CH_2Cl_2/MeOH extract of the leaves (10.24 g) was coated on diatomaceous earth (Dicalite Speed-Plus) in a ratio of 1:10 by weight. This was slurried in hexane and packed over an equal volume of flash chromatography grade silica (Merck No. 9385). Four to five column volumes of each solvent were used. Hexane, $CHCl_3$, $CHCl_3$/5% Me_2CO, $CHCl_3$/20% Me_2CO, Me_2CO, and MeOH were used in sequence to elute the column batchwise. The $CHCl_3$ through 20% Me_2CO fractions had the majority of bioactivity and were combined to give 5.16 g.
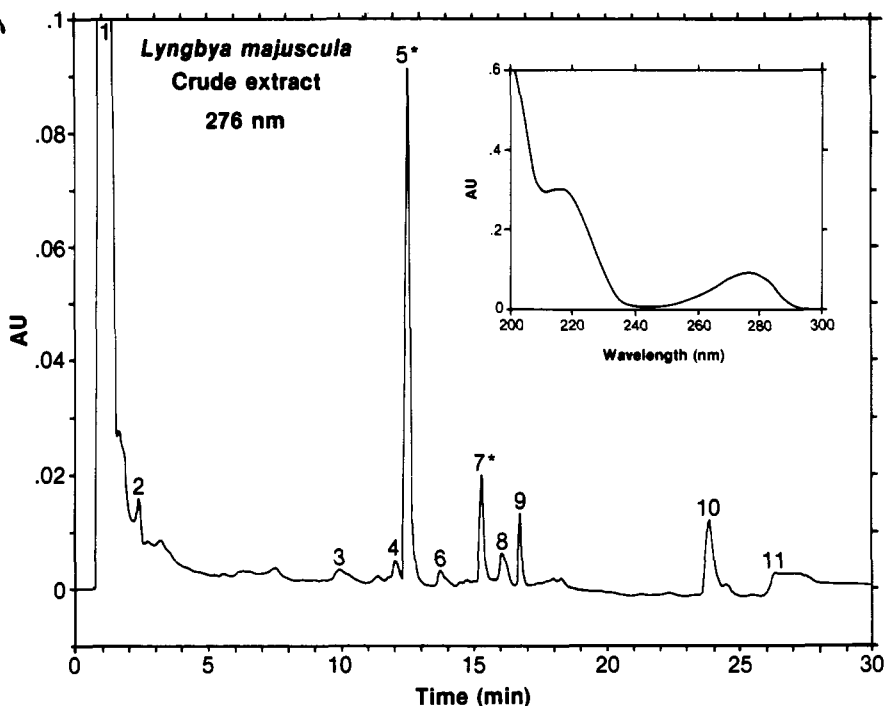
FIGURE 1.   *Lyngbya majuscula* crude organic extract, 10 μg injection on C_{18} Rainin Dynamax, 60Å, 3μ, 4.6 mm × 10 cm column in MeCN/H_2O gradient starting at 50% MeCN/2 min, 7-min linear gradient to 100% MeCN. Chromatogram detection at 276 nm, inset uv spectrum is for peak 5 (debromoaplysiatoxin). *Indicates PDBu bioactivity in peak.

This was rechromatographed in the same system with activity then localized in the 5% Me_2CO fraction (1.28 g, IC_{50} 0.3 μg/ml).

Flash chromatography (8) of the 5% Me_2CO fraction with a step gradient of Me_2CO in $CHCl_3$ gave fractions E (134 mg, IC_{50} <0.1 μg/ml) and F (178 mg, IC_{50} <1 μg/ml) (Figure 2). Fractions E and F were then separately chromatographed on C_{18} by hplc to give comparable fractions E-3 and F-3 which were back-extracted into $CHCl_3$ to give a total of 3.7 mg of active material (fraction G, IC_{50} 5 ng/ml). This material (1 μg) was rechromatographed at analytical scale and the eluate subjected to on-line PDBu binding (Figure 3). In the fabms of individual fractions from this chromatography a correlation was seen between bioactivity and presence of an *m/z* 311 peak. This peak was matched to a similar peak in standard PDBu run under the same conditions. Inactive apparent degradation products were located in fractions E-2 and F-2 (23.3 mg), which were combined and rechromatographed on C_{18} to afford 7.0 and 7.8 mg, respectively, of the purified compounds.

The highly active fraction G was hydrolyzed in 1 ml of 0.5 N KOH in MeOH for 30 min at room temperature, diluted with H_2O, and extracted into an equal volume of CH_2Cl_2. The residue was acetylated in pyridine/Ac_2O for 1.5 h at room temperature. The reaction mixture was quenched with H_2O, extracted into CH_2Cl_2, and evaporated. Upon examination by gc-ms as described above, three peaks were noted whose mass spectra resembled that of standard phorbol triacetate. The two larger of these peaks had $[M + NH_4]^+$ adducts at *m/z* 550, while the smallest of the three had an $[M + NH_4]^+$ adduct at *m/z* 536. All three of these peaks had extensive fragment ion similarity to standard phorbol triacetate (Figure 4).

RESULTS AND DISCUSSION

LYNGBYA.—The site 1 collection of *L. majuscula* was highly PDBu active, whereas the collection from site 10 was inactive. The crude extract of the active collection was examined directly by hplc-uv and found to contain large quantities of debromoaplysiatoxin (retention time and uv spectrum vs. standard). The PDBu activity was located by binding assay (Figure 1) in two uv absorbing peaks, one of which was debromoaplysia-
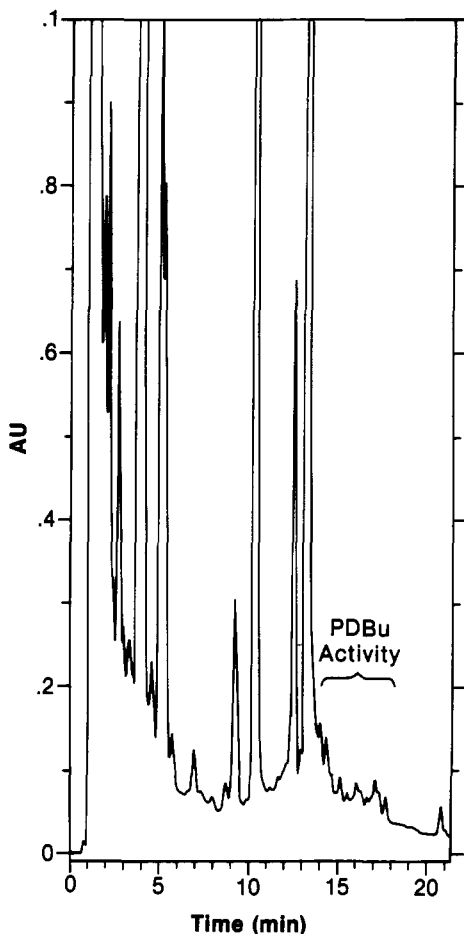
FIGURE 2.   *Croton cuneatus* fraction E chromato-
graphed on C_{18} Rainin Dynamax,
60Å, 3μ, 4.6 mm × 10 cm column
in MeCN/H_2O gradient starting at
50% MeCN/2 min, 7-min linear gra-
dient to 100% MeCN. Detection is
at 200 nm.

toxin. The other peak had a similar uv spectrum. No further active peaks were detected when larger amounts of extract were chromatographed. This method appears sensitive enough to detect a hundredfold weaker activity, if present.

Because the other taxa from the same site had lesser bioactivity, we explored methods of concentrating the bioactivity. An attempt to purify the crude extract of *Lyngbya* by batch elution over Si gel recovered only 4% of the PDBu activity. To cir-cumvent this acid/silica instability, we used diol phase-bonded material, which has been suggested as a substitute for Sephadex LH-20 (11), used previously in the isolation of debromoaphysiatoxin (3). By eluting with nonacidic organic solvents we concen-trated bioactivity and obtained high mass recovery, with a similar polarity profile to the silica elution. The purified fraction from diol media was examined with on-line PDBu binding, and activity was once again localized in the same two peaks. Diol media may be generally useful as a substitute for silica in rapid batch elution of compounds which are sensitive to silica.
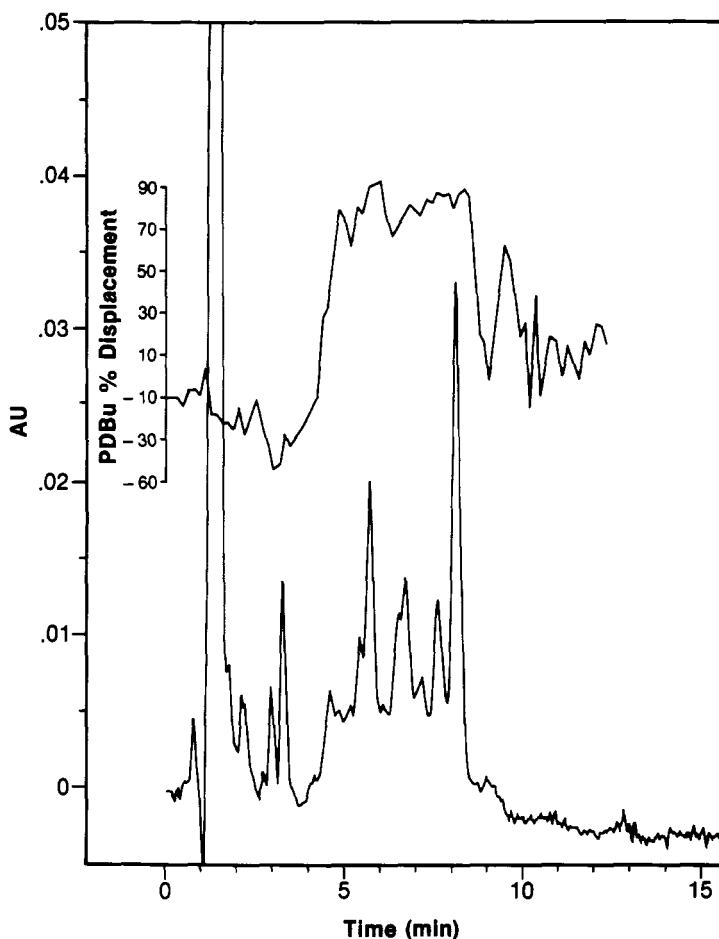
FIGURE 3.   *Croton cuneatus* fraction E-3, 1 μg injection, isocratic 80% MeCN, C$_{18}$ Rainin Dynamax, 60Å, 3μ, 4.6 mm × 10 cm column. Detection 190 nm.

We examined other collections from the same site that provided the bioactive *Lyngbya* and found that nine of twelve species of seaweeds and seagrasses had detectable PDBu bioactivity in their crude extracts (Table 1), although all were less potent than *L. majuscula*. The next most potent crude extract, from *Actinotrichea fragilis*, was subjected to hplc-uv analysis. Debromoaplysiatoxin was also conclusively identified in this sample by retention time and uv spectrum without further concentration. The aplysiatoxins are believed to be produced by microscopic blue-green associates of the macroalgal, filamentous *Lyngbya* (3). These other bioactive plants might also have the same blue-green algae associated with them, or the collection may have been contaminated with *Lyngbya*.

CROTON.—The leaf extract of *C. cuneatus* was the most potent plant part (IC$_{50}$ of 0.74 μg/ml); therefore it was selected for further examination. Bioactivity was concentrated by batch elution chromatography from diatomaceous earth, and rechromatography on silica yielded two potently active fractions. Analytical hplc resolved seven major peaks in both of these fractions when monitored at 190 nm (Figure 2); however, the PDBu binding assay (2 drop fractions) showed that none of the major uv-absorbing peaks was bioactive. Instead, bioactivity appeared to be associated with a series of very

TABLE 1.   PDBu-Binding Activity of Marine Plants Collected at Site 1.

| Plant Name	Percent Displacement [3]PDBu		IC$_{50}$ (µg/ml)
	100 µg/ml	10 µg/ml	
Actinotrichea fragilis (Forsskal) Borgesen	99	93	3.2
Amansia glomerata C. Agardh	95	53	~10
Ceratodictyon spongiosum Zananardi	92	—	16.2
Galaxaura oblongata (Ellis & Solander) Lamouroux	98	78	<10
Halodule uninervis (Forsskal) Ascherson[a]	81	2	~50
Halophila ovalis (R. Brown) Hooker f.[a]	22	−11	>100
Laurencia cartilaginea Yamada	37	−15	>100
Laurencia papillosa (C. Agardh) Greville	79	−6	~50
Lyngbya majuscula (Dillwyn) Harvey	112	101	0.29
Mastophora rosea (C. Agardh) Setchell	82	−1	~50
Microdictyon agarhianum Decaisne	98	60	<10
Portiera hornemanii (Lyngbe) P.C. Silva	86	26	~50
Syringodium isoetifolium (Ascherson) Dandy[a]	63	−8	~100

[a]Sea grass.

small peaks eluting just after the last major peak. Preparative scale hplc gave a potently active fraction (IC$_{50}$ 5 ng/ml), consisting of seven peaks by hplc, all of which appeared to have potent PDBu activity (Figure 3). On standing the active fraction yielded a pair of more polar products that were PDBu-inactive.

The diode array detector disclosed peaks with absorption maxima in the 190–200 nm range, as well as several with an additional chromophore at 230 nm typical of the α,β-unsaturated ketone of phorbol esters in the bioactive region of the chromatogram. Fabms of the active fraction showed a relatively clear spectrum with several features in common with standard PDBu. A prominent ion (50%) at m/z 311 from the active fraction was attributed to loss of the side chains and three molecules of H$_2$O (9, 10). Double loss of 30 amu (CH$_2$O) from putative molecular ions was also common to mass spectra of both PDBu and the active fraction. The combination of potent PDBu bioactivity, uv spectra, and mass spectral evidence led us to conclude that the bioactivity of *C. cuneatus* is due to unstable but conventional phorbol derivatives with saturated ester side chains.

When the apparent degradation products were examined by nmr, however, they were found to be acyclic C$_{20}$ isoprenoid alcohols similar to farnesyl alcohol (data not shown) instead of degraded phorbol diterpenes. What appeared to be degradation can be explained instead as $E{\mapsto}Z$ interconversion of the C$_{20}$ isoprenoid. The ^{1}H-nmr spectrum of the active fraction showed great similarity to the spectra of the inactive compounds, indicating that the active compounds in the fraction are masked by the presumably inactive C$_{20}$ isoprenoid. The active fraction was hydrolyzed, acetylated, and
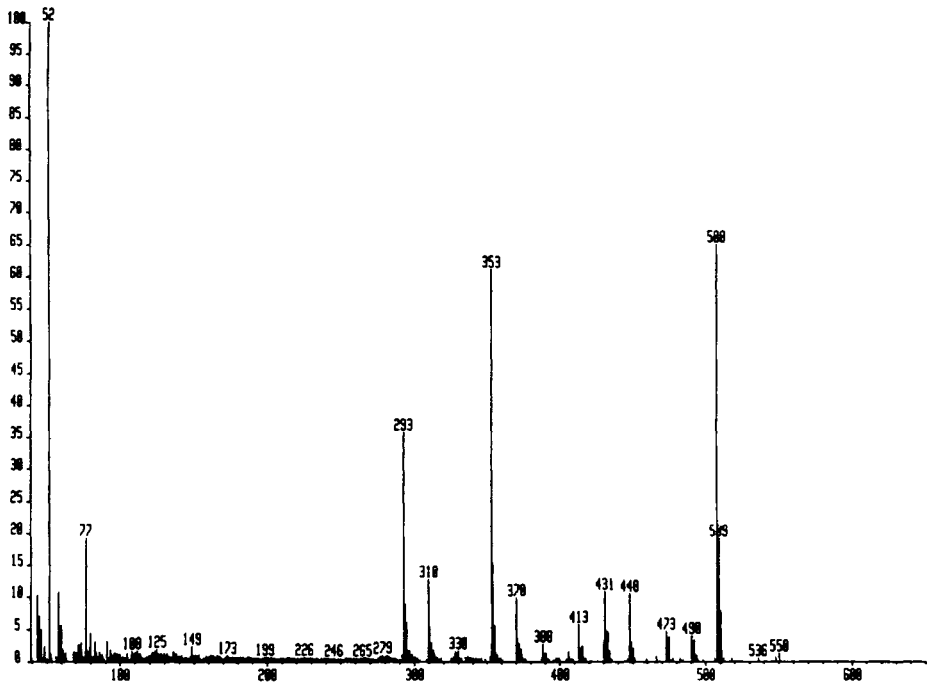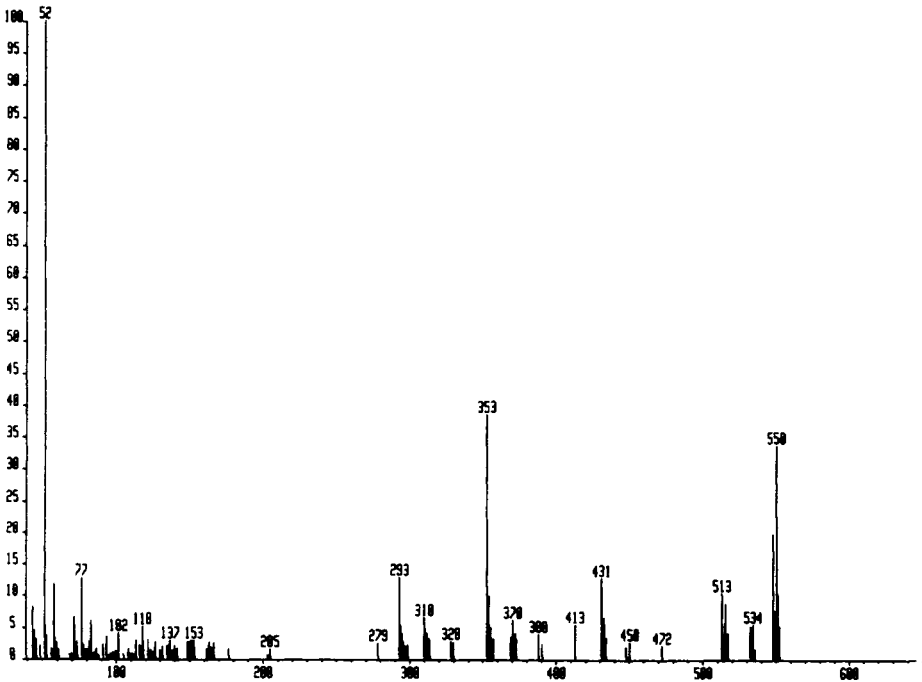
FIGURE 4.   Ci mass spectra of (a) standard phorbol triacetate and (b) gc peak from *Croton cuneatus* active fraction after hydrolysis and acetylation (see Experimental section).

examined by gc-cims using standard methods (12,13). Two minor peaks were identified as phorbol tetraacetates by comparison to phorbol triacetate synthesized from standard material. Figure 4 shows the comparison of one of these peaks to the standard. The parent diterpene alcohol can be identified as being in the phorbol series with a fourth hydroxyl accessible to acetylation: e.g., 4α-OH or 16-OH (14,15). It is not possible to determine the site of the extra hydroxyl by this method.

The PDBu assay combined with hplc-uv is a rapid and sensitive method for examining active samples and quickly determining if they contain known or new chemotypes. We have characterized these samples by hplc retention time, uv spectrum, and PDBu binding activity. Although direct uv and PDBu detection of debromoaplysiatoxin in the *Lyngbya* crude extract was possible, the small amounts of active phorbol esters in the *Croton* crude extract required prepurification to obtain a sample for gc-ms identification. Diol media was found useful for prepurification of extracts such as *Lyngbya* which are sensitive to silica. Actual isolation of pure material and extensive characterization by nmr are unnecessary, and the scale of isolation work can be kept small.

Dereplication of PDBu-active crude extracts by these methods makes it possible to characterize the structural types and in some cases identify particular compounds responsible for the activity. The *Croton* example shows that caution must be taken when interpreting such data, because small amounts of a potent bioactive compound can co-elute with inactive material even after several chromatography steps. Nevertheless, the rapidity of the method should permit dereplication of all of the PDBu-active crude extracts with a reasonable amount of effort. These methods appear to be generally applicable to organic extracts from terrestrial and marine plants, marine animals, and microbial sources.

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