The Journal of
Organic
Chemistry

VOLUME 58, NUMBER 16 JULY 30, 1993

Communications

Synthesis and Binding of Photoaffinity Ligand Candidates for Protein Kinase C

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Received March 30, 1993

Summary: Our observation that protein kinase C binding is retained by phorbol esters modified at the C-3 position has led to the design of a new class of photoaffinity ligand candidates. The first members of this class, esters **3** and **4**, were synthesized from phorbol and found to bind to PKC with high affinity; the solution photochemistry of **3** leads predominantly to insertion products, as required for its use as a PKC receptor probe.

Protein kinase C (PKC) is a phospholipid-dependent serine/threonine kinase which plays a fundamental role in cellular signal transduction.[2] It is especially noteworthy that PKC is also the primary target of tumor-promoting phorbol esters (PEs: Scheme I, **1b**).[2] While recent studies have demonstrated that a cysteine-rich tandem repeat in the PKC regulatory domain is necessary and sufficient for PE binding,[3,4] the specific amino acids in this sequence with which PEs interact have not been identified. Photoaffinity labeling is a promising approach to solve this problem and the related goal of establishing the solution structure of the regulatory domain of PKC. Thus far, however, attempts to label PKC with photolabile PEs have resulted in labeling of only the surrounding phospholipid.[5,6] Our previous structure–activity and molecular modeling studies[7a,h] suggest that these results could be attributed to the positioning of the affinity label at a site (C-12) in the PEs that is on the surface of the PKC–PE complex. Since these studies[7a,h] also indicate that oxygens at C-9, C-20, and C-3 or C-4 of the PEs interact with PKC, attachment of a photolabile group at or near one of these PE sites should allow for photoaffinity labeling of PKC. On the basis of our finding that 3β-hydroxyphorbol ester **2a** shows significant PKC binding,[8] we now report the synthesis of PEs **3** and **4**, members of a new class of photoaffinity probes for PKC.

The syntheses of **3** and **4** started with the conversion of phorbol (**1a**) to its triester **1c** (43%).[9,10] Sodium borohydride reduction of **1c** in the presence of cerium(III) chloride, followed by treatment with tetra-*n*-butylammonium fluoride (TBAF), gave exclusively one alcohol stereoisomer (**2a**, 78%), which was assigned the 3β-hydroxy stereochemistry on the basis of mechanistic considerations and its conversion to a C-3, C-4 acetonide.[8] For comparison purposes, the complementary stereoisomer **2b** was produced (58%) by reduction of **1c** with sodium triacetoxyborohydride.[8,11] These assignments were confirmed by

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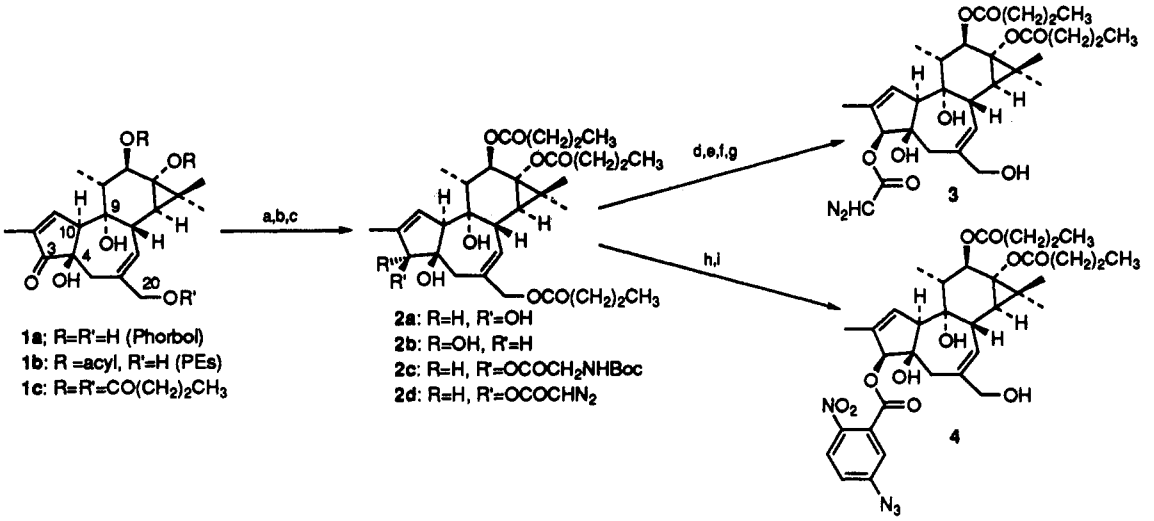
(9) Caution: Phorbol esters are potent tumor promoters and should be handled with care.

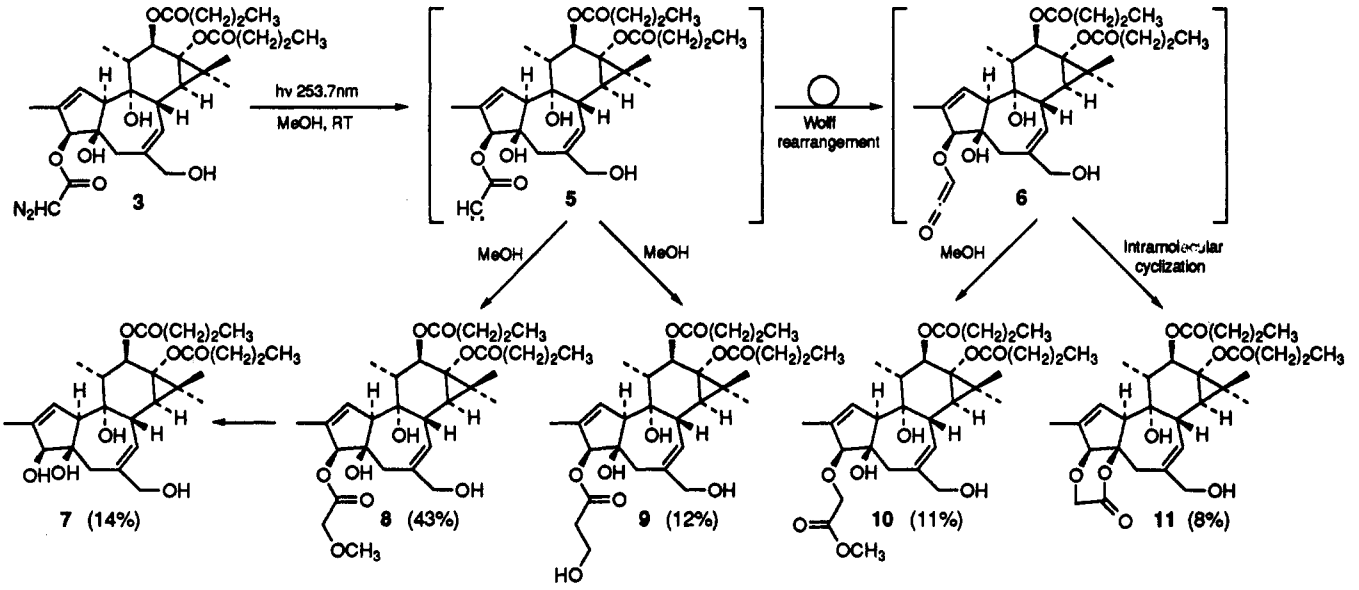
0022-3263/93/1958-4179$04.00/0

Scheme I[a]



[a]Key: (a) $(CH_3CH_2CH_2CO)_2O$, DMAP, triethylamine, CH_2Cl_2; (b) $NaBH_4$, $CeCl_3$, MeOH; (c) TBAF, THF; (d) Boc-Gly, DMAP, triethylamine, DCC, THF; (e) 1 N HCl, acetic acid; (f) $NaNO_2$, H_2O–CH_2Cl_2, $H^+(H_2SO_4)$; (g) $Ba(OH)_2$, MeOH; (h) 5-nitro-2-azidobenzoic acid, DMAP, triethylamine, DCC, THF; (i) $HClO_4$, MeOH. II

Scheme II



NOE difference spectroscopy of **2a** and **2b** in deuterochloroform. Saturation of the C-3 proton (δ4.21) in **2a** caused a remarkable enhancement (22%) of the C-10 proton signal (δ2.98) while no enhancement was detected for this proton in isomer **2b**.

While attempts to convert alcohol **2a** to the diazoacetyl derivative **3** via the glyoxylic acid *p*-toluenesulfonylhydrazone[12] were frustrated by the lability of intermediates,[13] an alternative route based on glycine proved to be effective.[14] Alcohol **2a** was esterified with Boc-glycine in the presence of DCC to give the glycinate **2c** (78%). The Boc group of **2c** was then cleaved by 1 N HCl in acetic acid. Treatment of the resulting amine with sodium nitrite and sulfuric acid in water–dichloromethane provided the diazoester **2d** (40% for 2 steps). While acidic hydrolysis of the C-20 ester of **2d** was precluded by competitive loss of the diazoacetyl group, the selective hydrolysis of this tetraester to triester **3** was eventually achieved by using barium hydroxide in methanol. The synthesis of the azido derivative **4** was readily accomplished by condensation of **2a** with 5-azido-2-nitrobenzoic acid in the presence of DMAP, triethylamine, and DCC and selective hydrolysis of the resultant tetraester with perchloric acid in methanol to provide **4** (22% for two steps).

As a prelude to PKC affinity-labeling studies, the photolytic fate of these probes and their ability to serve as trapping agents were investigated. For this purpose, a room-temperature, methanol solution of **3** (1 mg/mL; Scheme II) under a nitrogen atmosphere was irradiated at 253.7 nm (Rayonet photochemical reactor). Photoconversion was complete within 5 min and gave one major

(10) The structures of all compounds (>98% purity) were confirmed by IR, UV where relevant, ^1H NMR, and high-resolution FAB-MS.

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product and four minor products in isolated yields of 43%, 14%, 12%, 11%, and 8%, respectively. The major compound (8) proved to be the desired solvent trapping (insertion) product. Alcohol 7 presumably results from hydrolysis of the ester group at position 3 upon photolysis and/or during the purification steps. Compound 9 was also identified as a solvent-trapping product, arising from carbene insertion into the CH bond of methanol. Compounds 10 and 11 presumably arise from Wolff rearrangement of the initially formed carbene followed by intra- and intermolecular addition to the resulting ketene. Although a diazoacetyl group generally suffers as an affinity label due to its propensity to undergo Wolff rearrangement, the photolytic behavior of 3 involving >50% solvent insertion bodes well for its service as a photoaffinity label for PKC. Photolysis of 4 in methanol at 300 nm yielded a large number of unidentifiable, polar degradation products. However, because the photochemical behavior of 4 in the PKC-bound state might be substantially different, these results do not necessarily disqualify it as a photoaffinity ligand.

The further development of 3 as an affinity label requires that it eventually be isotopically labeled. The feasibility of this labeling was determined by oxidation of 3 with activated MnO_2 to give the C-20 aldehyde and subsequent $NaBH_4$ reduction on a scale required for tritium handling (900 μg). The yield of cold 3 in this feasibility run was 52%, strongly suggesting that tritium-labeled 3 can be synthesized by this procedure using $NaBT_4$. Compound 3 could also be labeled with ^{14}C using ^{14}C-labeled glycine.

The final and crucial factor bearing on the utility of 3 as a photoaffinity probe for PKC, namely, its ability to bind PKC, was determined by using partially purified rat brain PKC. This assay was carried out by using the method of Tanaka,[15] a rapid filtration procedure in which a glass-fiber filter pretreated with a cationic polymer, polyethylenimine, is used to trap the enzyme–substrate complex. Scatchard plot analysis using [^{3}H]phorbol-12,13-dibutyrate (PDBu) indicated that in our hands this assay gave a K_d value for PDBu binding of 2.6 nM (i.e., similar to that previously reported).[15] Table I shows the inhibition of specific [^{3}H]PDBu binding to PKC by the two photoaffinity probes. The binding affinity was evaluated by the concentration required to cause 50% inhibition, IC_{50}. The two photoaffinity probes significantly bound PKC, and while their binding was 10–100-fold weaker than PDBu, it is sufficient for their use in the photoaffinity labeling of PKC. Studies on the labeling of PKC are in progress.

Table I. Inhibition of Specific [^{3}H]PDBu Binding to PKC[a]

compd	IC_{50} (nM)	K_i (nM)
3	693.2	141.5
4	46.3	9.5
1,2-dioctanoyl-*sn*-glycerol	1126.0	229.0
PDBu	4.9	1.0

[a] The binding assay was carried out in 300 μL of buffer containing 50 mM Tris–HCl (pH 7.5), 4 mg/mL of bovine γ-globulin, 10 nM [^{3}H]PDBu, 50 μg/mL of phosphatidylserine, 2 mM $CaCl_2$, and 14 μg/mL of partially purified rat brain PKC. The IC_{50} values were calculated from triplicate experiments.

Acknowledgment. This research was supported by a grant (CA31841) from the National Institutes of Health and fellowship support (K.I.) from the Uehara Memorial Foundation. We thank Professor Wray H. Huestis and Mr. Mike Moxness for a sample of partially purified rat brain PKC and for the use of their radioisotope facilities.

Supplementary Material Available: Experimental procedures and analytical data for all new compounds (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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