*Pure & Appl. Chem.*, Vol. 70, No. 3, pp. 539–546, 1998.
Printed in Great Britain.
© 1998 IUPAC

# The chemistry–medicine continuum: Synthetic, computer, spectroscopic and biological studies on new chemotherapeutic leads

Paul A. Wender*, Yolanda Martin-Cantalejo, Andrew J. Carpenter, Anna Chiu, Jef De Brabander, Patrick G. Harran, Juan-Miguel Jimenez, Michael F.T. Koehler, Blaise Lippa, James A. Morrison, Stephan G. Müller, Stephan N. Müller, Cheol-Min Park, Makoto Shiozaki, Carsten Siedenbiedel, Donald J. Skalitzky, Masahiro Tanaka, and Kazuhiro Irie[a]

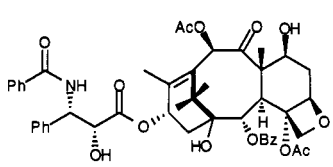
*Department of Chemistry, Stanford University, Stanford, CA 94305-5080, USA*

[a]*Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan*

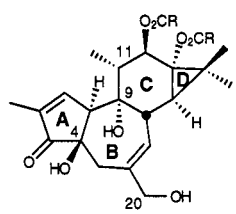
*Abstract* – This lecture provides an overview of investigations directed towards understanding the molecular mechanism of protein kinase C (PKC) activation and function. Central to this effort are studies on the total synthesis of phorbol, the first asymmetric synthesis of phorbol, and the first synthesis of resiniferatoxin, all involving highly effective applications of [5+2] oxidopyrylium-alkene cycloadditions. The synthesis and affinities of the phorbol ester binding domain of PKC are also presented. In addition, a pharmacophore model for agonist binding to PKC is presented in connection with the design of novel PKC activators. Finally, the computer modeling, NMR structure, synthesis, and biological activity of the first fully synthetic bryostatin analogs are described.

Studies in our laboratory focus on the synthesis and investigation of molecules of structural, biological, and medicinal significance. Representative of this program are compounds (Scheme 1) such as taxol (**1**),[1] phorbol esters (**2**),[2] and bryostatin (**3**).[3] These fascinating molecules have been the focus of intense research activity over the past several decades and have provided the stimulus for major advances in chemistry, biology, and medicine. Taxol is now used clinically for the treatment of breast and ovarian cancers. Phorbol esters serve as molecular probes for the investigation of how cancer starts and consequently of how it might be prevented. They also serve as leads for the study of cellular signal transduction and for the development of new medicinal agents. Bryostatin is now in several phase II clinical trials as a cancer chemotherapeutic agent. In addition, it has been found to provide protection against normally lethal doses of ionizing radiation and has novel immune stimulation properties, effecting the production of interleukin and interferon.

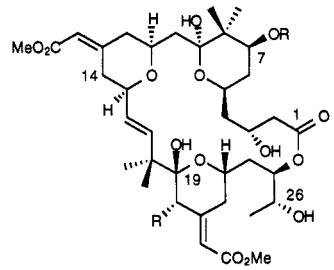
**Scheme 1:** Representative Structures of Synthetic, Biological, and Medicinal Interest




Taxol (**1**)




Phorbol Esters (**2**)



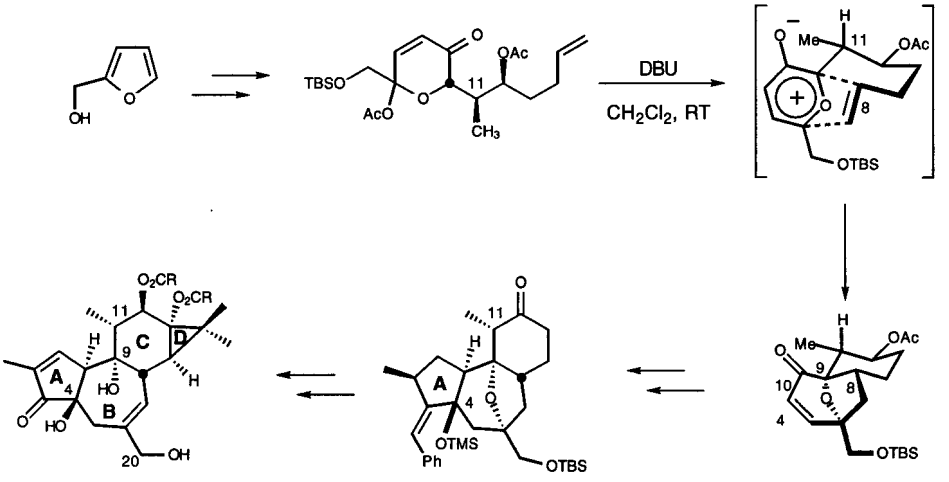

Bryostatin (**3**)

Our interest in these and related compounds is driven by the paucity of information on their molecular mechanism of action, the formidable challenges associated with their syntheses, and the potential value that such synthetic and mechanistic studies might have on the prevention, diagnosis, and treatment of cancer as well as other diseases. This lecture will provide an overview of computer, synthetic, spectroscopic, and biological studies on phorbol and bryostatin, as well as their receptors, protein kinase C isozymes, key enzymes which mediate cellular signal transduction.[4]

The phorbol esters are the most potent tumor promoters known. They exhibit a remarkable ability to amplify the effect of a carcinogen but are themselves not carcinogenic. This unusual activity has been the focus of great research interest over the past half century and has provided the basis for the development of the multistage theory of carcinogenesis. More recently, it has been proposed that tumor promotion and other biological activities of the phorbol esters arise through their potent activation of PKC.[5] The development of an understanding of the molecular mechanism of action of phorbol esters and other tumor promoters and the role of PKC in this process are central to understanding carcinogenesis at the molecular level and thus to developing prevention protocols for cancer.

In order to gain an understanding of the structural basis of phorbol's unique biological effects, the total synthesis of phorbol and access to its analogs became central objectives in our program. The synthetic challenge associated with this class of molecules is formidable as suggested by the presence in phorbol of 8 stereogenic centers on 4 different and densely functionalized rings, the absence of synthetic precedent in this area at the outset of our studies, the finding that these compounds are sensitive to acid, base, heat, and light, and the fact that they are biohazards at nanomolar concentrations. We reported the first racemic synthesis of phorbol in 1989,[2a] and an asymmetric synthesis was recently completed,[2d] both based on a [5+2] oxidopyrylium-alkene cycloaddition to form simultaneously the BC-ring system and the C8 and C9 stereogenic centers of phorbol (Scheme 2). A major emphasis in these studies has been placed on the development of novel and general synthetic strategies and methods for accessing related biologically active analogs. This philosophy has recently also allowed for the adaptation of the oxidopyrylium-alkene cycloaddition to the first asymmetric total synthesis of resiniferatoxin (RTX, Scheme 3),[6] an ultrapotent analog of capsaicin and the most potent irritant known.[7] The synthetic expertise gained in these studies is currently being used to elucidate the structural basis for the activities of the phorbol esters, RTX, and their analogs, to identify their receptors, and to determine the detailed molecular features associated with receptor-substrate recognition.

**Scheme 2:** Key Strategic Stages in the Asymmetric Synthesis of Phorbol Esters

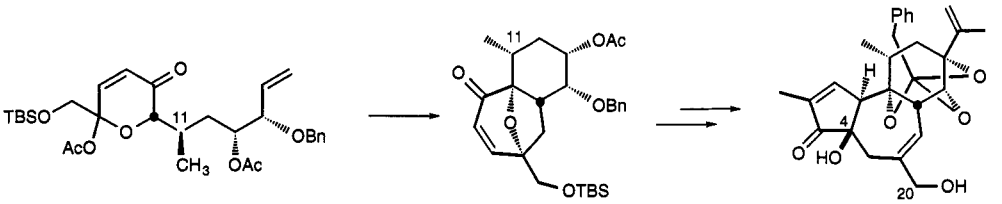



Phorbol Esters (**2**)

Concurrent with the above studies, we began to focus on the structural characterization of PKC itself, the receptor for diacyl glycerol, phorbol esters, and putatively for bryostatin as well. This information is of great value in efforts to understand normal and abnormal cell function mediated by the binding of diacyl glycerol and phorbol esters, respectively, to PKC. Both bind

to PKC but the plant derived phorbol esters bind with affinities up to $10^3$ greater than diacyl glycerol, the endogenous activators of this mammalian receptor, and clearly induce a dramatically different set of downstream consequences.

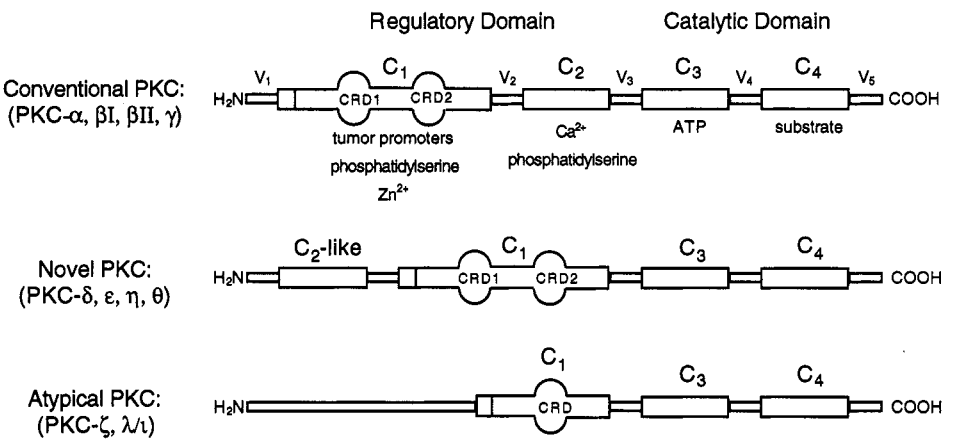
**Scheme 3:** The Oxidopyrylium [5+2] Cycloaddition in the First Synthesis of Resiniferatoxin




Resiniferatoxin

Information about the structure of PKC has been limited thus far by its large size, which makes NMR solution structure analysis impossible at present, and its requirement for phospholipid cofactors, which renders it difficult to obtain PKC-phospholipid aggregate crystals suitable for X-ray crystallography. To date, at least 11 PKC isozymes have been identified and classified into 3 groups based on their structure and cofactor requirements (Scheme 4).[8] The first isozymes to be discovered were the so called *conventional* PKCs (PKC-α, βI, βII, and γ), which have been shown to contain four conserved domains ($C_1$-$C_4$), and five variable ($V_1$-$V_5$) regions. Conventional PKCs consist of a catalytic domain for protein phosphorylation ($C_3$ and $C_4$), and a regulatory domain ($C_1$ and $C_2$) which binds phorbol esters and calcium in the presence of phosphatidylserine. A second group of PKC isozymes is referred to as the *novel* class (PKC-δ, ε, η, and θ). They are structurally similar to the conventional class, except for the absence of the $C_2$ region and, consequently, calcium dependent binding. Within the regulatory domain of conventional and novel PKC's are conserved cysteine rich domains (CRD's) which are implicated in phorbol ester and diacyl glycerol binding to and activation of PKC. The least understood class of isozymes is the *atypical* PKC's which do not bind phorbol esters.

**Scheme 4:** Classifications of Protein Kinase C (PKC) Isozymes



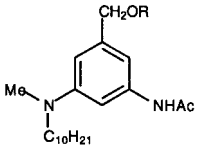

Given that efforts to characterize complete PKC isozymes have been hampered by their size and aversion to forming suitable crystalline phospholipid aggregates, we initiated an effort several years ago aimed at identifying the smaller regulatory domain of PKC's, i.e., the region responsible for the binding and implicated in initiating the biological activities of the tumor promoting phorbol esters, the chemotherapeutic candidate bryostatin, and the endogenous PKC activator diacyl glycerol. Our approach rested on the view that information on the solution structure of the phorbol ester binding site could be obtained from these much smaller and more readily available regulatory domain subunits rather than the entire protein itself. This approach would of course be useful only if the regulatory domain subunits folded in the same way as the whole protein and exhibited the same recognition capability.

To test this approach, cysteine rich domains (CRDs) of the regulatory region of PKC were prepared by recombinant DNA techniques and solid phase synthesis.[9] The former approach

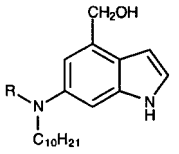
allows for great variation in the sequence produced while the latter can be used to prepare relatively large quantities of the desired sequences. Using this approach, it was found that these simple subunits of PKC do indeed bind phorbol esters and even exhibit a phospholipid dependence like PKC itself, even though they represent only 10-15% of the protein. A collaborative effort with the group of Kazuhiro Irie (Kyoto University) has more recently shown that the magnitude of the binding of phorbol esters to some regulatory domain subunits approaches that of the whole protein itself, making these surrogates excellent models for the study of the interaction of phorbol esters and other molecules with the regulatory domains of PKC isozymes. Further studies have led to the synthesis of both the CRD1 and CRD2 from PKC-γ, a conventional isozyme, and PKC-η, a novel isozyme. The availability of these protein surrogates has allowed for an evaluation of the extent to which each CRD is responsible for binding and function, a starting point for the design and development of isozyme specific inhibitors and activators. Representative of the findings derived from these novel probes, the CRD1 of PKC-η binds tritiated phorbol 12,13-dibutyrate (PDBu) in the presence of zinc chloride and phosphatidylserine with a $K_d$ of >200 nM while the CRD2 surrogate binds with a $K_d$ of 0.91 nM. In accord with the remarkable similarity of these surrogates and the native isozymes, PDBu binding with pure PKC-η has a $K_d$ of 0.87 nM.[10] It is expected that these CRDs, which retain the phorbol binding characteristics of native PKC, will serve as molecular probes for the determination of the structural requirements of normal and abnormal PKC activation and for the development of new medicinal leads based on this receptor. In addition, they provide new options for high throughput isozyme specific assays and avoid the reliance on animal sources for isozymes.

In a related effort, we had initiated a computer study of known tumor promoters and PKC activators in order to gain a more thorough understanding of how phorbol and other tumor promoters bind to PKC. Such information could potentially be of value in the development of prevention protocols for cancer, since it could be used as a rapid first screen for the identification of tumor promoter candidates. It is also of value for the design of selective activators and inhibitors of the isozymes, which due to their different functions could in principle be selectively regulated. We proposed a pharmacophore model for PKC agonists that could be used to rationalize how structurally dissimilar molecules like phorbol, teleocidin, gnidimacrin, ingenol, and 1,2-diacyl-*sn*-glycerol (DAG), the endogenous PKC activator, might bind to the same site or similar sites on PKC.[11] In this study, the interatomic distances of all possible heteroatomic triads in the relatively rigid exogenous agonists were compared with heteroatomic triads in low energy conformers of DAG. The best correlation from this analysis indicated that the C4, C9, and C20 hydroxyls of phorbol played a principle role in phorbol recognition by PKC. Spatially and functionally analogous heteroatomic triads were also found in all agonists suggesting that this array could be a common determinant of PKC binding. Other correlations were also identified and ranked from comparable to irrelevant, providing a series of structural hypotheses for evaluation. Notably, this information was key in the design and the *de novo* synthesis of several molecules exhibiting affinities for PKC comparable to diacyl glycerols themselves (Scheme 5). Moreover, these first designed PKC activators were also shown to function *in vitro*, inducing phosphorylation in 3T3 cells and human platelets.

**Scheme 5:** The First Designed PKC Activators




R=H or R=Ac




R=H or R=Me

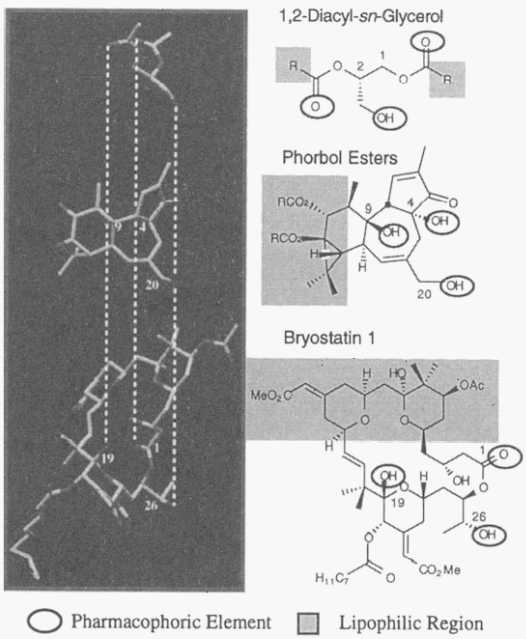
After it was reported that the bryostatins bind with high affinity to PKC,[12] the groups of Wender, Pettit, and Blumberg investigated the possible structural similarities between phorbol, diacyl glycerol, and bryostatin 1 in an effort to determine whether they possess similar recognition subunits.[13] It should be emphasized that the competitive binding exhibited by these compounds does not require that they possess similar recognition subunits. It is also important to note that they could interact differently with functionality in the receptor domain. However, the hypothesis that these molecules could share a common pharmacophore (recognition unit) which is recognized similarly in the receptor domain was seen as an important starting point for addressing the nature of the recognition process. Computer modeling subsequently revealed a rather good correlation between the C1 carbonyl, C19 hydroxyl, and the C26 hydroxyl triad of bryostatin 1 and related triads in phorbol esters and diacyl glycerol (Scheme 6). The resulting

hypothesis was that the A and B rings of bryostatin serve partly as spacers to remotely control the orientation and mobility of the groups (C1, C19, and C26) putatively required for recognition. This hypothesis was consistent with the then known bryostatin structure activity relationships and continues to be in agreement with recent studies on newly discovered bryostatins. This hypothesis has now been used in the design of the first simplified, fully synthetic bryostatin analogs which retain PKC binding affinity and, in the case examined, possess significant levels of *in vitro* cell growth inhibitory activity.

Due to the limited availability of the natural bryostatins and the difficulties encountered in their selective modification, structure-activity studies have thus far been largely confined to the natural products themselves and closely related derivatives. These studies have however revealed some information pertinent to the design of analogs. Comparison with the PKC binding affinities of bryostatin 1 to modified structures, both natural and semi-synthetic, indicate that modifications in the A and B rings have only modest effects, while modifications in the C ring and especially the C26 alcohol have rather dramatic effects. These structure-activity relationships and computer modeling of the bryostatins suggest that bryostatin-like recognition could be achieved in analogs which retain the natural functionality in the putative recognition domain (C17-C27, and C1) but incorporate simplified spacers (C2-C16) (Scheme 7).

In order to rapidly evaluate the conformational correspondence between the designed analogs and bryostatin, the conformations of the analogs were first determined by molecular mechanics calculations using the multiconformer mode of the MM2 force field provided with Macromodel® v.4.5 and compared with the known solid state and solution structures of the bryostatins. Ten thousand analog conformations were randomly generated and minimized using a truncated Newton conjugate gradient. Water solvation was simulated by the generalized Born/ solvent accessible surface area model.[14] This modeling suggested that simplified analogs of type **4**, devoid of functionality along the C7-C13 periphery of the molecule and incorporating an oxygen in place of C14 to facilitate synthesis, had a conformation very similar to the natural product.

**Scheme 6:** Correlation of Recognition Elements in 1,2-Diacyl-*sn*-glycerol, Phorbol Esters, and Bryostatin 1



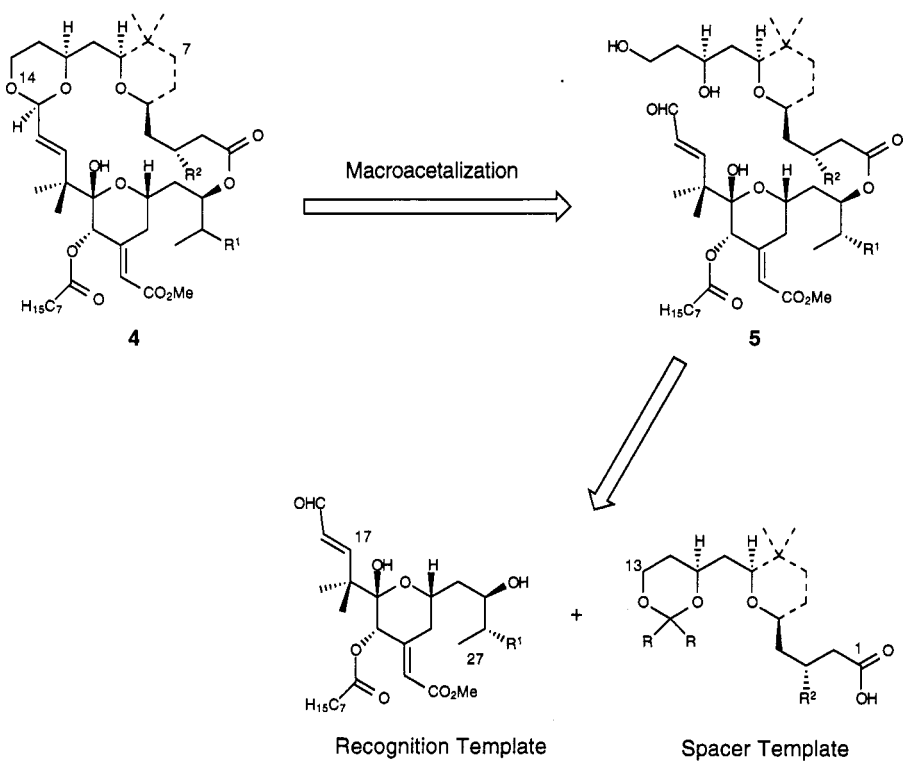

Novel bryostatin analogs **6a,b** and **7** were synthesized according to the general strategy outlined in Scheme 7. This allowed maximum convergency at a late stage of synthesis, and importantly, only three steps are needed for the final coupling of the bryostatin and spacer templates and analog completion. Similarly prepared were acetate **6c** and C26 epimeric alcohol **6d**, which would be expected to function as negative controls, analogous to the corresponding bryostatin derivatives which do not bind PKC with high affinity.[15] Another

feature of interest in this synthesis is the method of macrocycle closure involving a novel macrotransacetalization.

As a cross check of our molecular modeling, and as a final screen to identify potential analog candidates, an NMR structure of analog **6a** was sought. Phase sensitive NOESY and DQF-COSY spectra of compound **6a** in $C_6D_6$ were analyzed in order to determine the identity and categorical distances between protons throughout the structure. From this analysis, 43 unique internal distance constraints were generated and subsequently simulated in constrained gas-phase molecular dynamics followed by minimization. This identified a group of low energy conformers which compared favorably with the published crystal structure of bryostatin 1 and the solution structure of bryostatin 10. The RMSD calculated by superimposing the putative pharmacophoric atoms of the lowest energy conformer onto their counterparts in bryostatin 1 is 0.181 Å, indicating a remarkably good correlation between the two structures. Thus, computer modeling and NMR structural analysis of the designed analogs suggested that they emulate bryostatin rather well.

In accord with the computer and NMR analyses, analog **6a** exhibited excellent affinity for PKC (see Scheme 8).[16] The broader value of our convergent assembly is immediately evident in the ease with which structurally diverse analogs can be prepared. For example, analog **6b** was also synthesized and found to possess similar activity to **6a**, indicating that the C3 hydroxyl is not important for activity at least in this analog series. In addition, analog **7** which is further simplified relative to **6a,b**, as a result of the strategic placement of a conformationally constraining *tert*-butyl group at C9 in lieu of the A ring of bryostatin, showed even better activity (89 nM). As expected, the C26 acetate **6c** and the C26 epimeric alcohol **6d** showed at best weak PKC binding affinity. In the one case studied thus far (analog **6a**), it was possible to conduct a functional assay involving growth inhibition against several human cancer cell lines. Significant activity was observed against all six cell lines studied ($GI_{50}$= 0.94-5.9 μg/mL).[17]

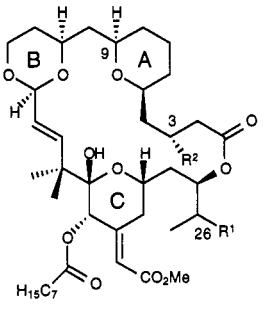
**Scheme 7:** The Design of Novel Bryostatin Analogs



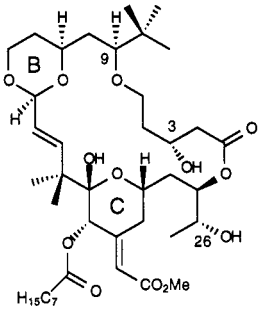

Overall this program has thus far produced a number of advances which provide the foundation for new developments in synthesis, new information on molecular recognition at the PKC regulatory domain, and novel leads of potential medicinal value. Further simplification of

the first designed bryostatin analogs represents a particularly exciting approach to the generation of clinically superior agents which could efficiently be produced through synthesis. Further development of these leads is under active investigation.

**Scheme 8:** PKC Binding Affinities of Designed Bryostatin Analogs and Control Compounds



**6a-d**



**7**

| Compound | $R^1$ | $R^2$ | $K_i$ (nM) |
|---|---|---|---|
| **6a** | αOH | OH | 285 |
| **6b** | αOH | H | 297 |
| **6c** | αOAc | OH | >20000 |
| **6d** | βOH | OH | >20000 |
| **7** | - | - | 89 |

**Acknowledgments:** Support of this work through a grant (CA31845) provided by the National Institutes of Health (P.A.W.), a Grant-in-Aid for Scientific Research on Priority Areas (No. 08219224), and Scientific Research (C) (No. 08660137) from the Ministry of Education, Science, and Culture, Japan (K.I.) are gratefully acknowledged. HRMS analyses were performed at the University of California, San Francisco Regional and the University of California, Riverside Mass Spectrometry Facilities. Fellowship support from the following institutions is also gratefully recognized: Fulbright / Spanish Ministry of Education and Science (J.M.J., Y.M-C.), American Cancer Society (A.J.C.), Bing Foundation (A.C.), Fulbright-Hays / NATO (J.D.B.), NIH (P.G.H., J.A.M.), Eli Lilly (M.F.T.K.), Deutsche Forschungsgemeinschaft (S.G.M.), Swiss National Science Foundation / Ciba-Geigy-Jubiläums-Stiftung (S.N.M.), the Korean Science and Engineering Foundation (C-M.P.), Japan Tobacco (M.S., M.T.), and the Alexander von Humboldt Foundation (C.S.).

## References

1. a) Wender, P. A.; Badham, N. F.; Conway, S. P.; Floreancig, P. E.; Glass, T. E.; Gränicher, C.; Houze, J. B.; Jänichen, J.; Lee, D.; Marquess, D. G.; McGrane, P. L.; Meng, W.; Mucciaro, T. P.; Mühlebach, M.; Natchus, M. G.; Paulsen, H.; Rawlins, D. B.; Satkofsky, J.; Shuker, A. J.; Sutton, J. C.; Taylor, R. E.; Tomooka, K. *J. Am. Chem. Soc.* **1997**, *119*, 2755-2756. b) Wender, P. A.; Badham, N. F.; Conway, S. P.; Floreancig, P. E.; Glass, T. E.; Houze, J. B.; Krauss, N. E.; Lee, D.; Marquess, D. G.; McGrane, P. L.; Meng, W.; Natchus, M. G.; Shuker, A. J.; Sutton, J. C.; Taylor, R. E. *J. Am. Chem. Soc.* **1997**, *119*, 2757-2758.
2. a) Wender, P. A.; Lee, H. Y.; Wilhelm, R. S.; Williams, P. D. *J. Am. Chem. Soc.* **1989**, *111*, 8954-8957. b) Wender, P. A.; Kogen, H.; Lee, H. Y.; Munger Jr., J. D.; Wilhelm, R. S.; Williams, P. D. *J. Am. Chem. Soc.* **1989**, *111*, 8957-8958. c) Wender, P. A.; McDonald, F. E. *J. Am. Chem. Soc.* **1990**, *112*, 4956-4958. d) Wender, P. A.; Rice, K. D.; Schnute, M. E. *J. Am. Chem. Soc.* **1997**, *119*, 7897-7898.

3. Wender, P. A.; De Brabander, J.; Harran, P. G.; Jimenez, J. M.; Koehler, M. F. T.; Lippa, B.; Park, C.; Siedenbiedel, C.; Pettit, G. R. *Proc. Natl. Acad. Sci. USA* submitted. b) Wender, P. A.; De Brabander, J.; Harran, P. G.; Jimenez, J. M.; Koehler, M. F. T.; Lippa, B.; Park, C.; Shiozaki, M. *J. Am. Chem. Soc.* submitted.
4. Nishizuka, Y. *Nature* **1984**, *308*, 693-698.
5. Castagna, M.; Takai, Y.; Kaibuchi, K.; Sano, K.; Kikkawa, U.; Nishizuka, Y. *J. Biol. Chem.* **1982**, *257*, 7847-7851.
6. Wender, P. A.; Jesudason, C.; Tamura, M.; Tebbe, A. L.; Ueno, Y.; Nakahira, H. *J. Am. Chem. Soc.* **1997**, *119*, in press.
7. Szallasi, A.; Blumberg, P. M. *Neuroscience* **1989**, *30*, 515-520.
8. For reviews, see: a) Marks, F.; Gschwendt, M. *Mutation Res.* **1995**, *333*, 161-172. b) Newton, A. C. *J. Biol. Chem.* **1995**, *270*, 28495-28498. c) Nishizuka, Y. *FASEB J.* **1995**, *9*, 484-496.
9. a) Yanai, Y.; Irie, K.; Ohigashi, H.; Wender, P. A. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 117-122. b) Wender, P. A.; Irie, K.; Miller, B. L. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 239-243. c) Irie, K.; Yanai, Y.; Ohigashi, H.; Wender, P. A.; Miller, B. L. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 353-356.
10. Kazanietz, M. G.; Areces, L. B.; Bahador, A.; Mischak, H.; Goodnight, J.; Mushinski, J. F.; Blumberg, P. M. *Mol. Pharmacol.* **1993**, *44*, 298-307.
11. Wender, P. A.; Koehler, K. F.; Sharkey, N. A.; Dell'Aquila, M. L.; Blumberg, P. M. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4214-4218.
12. De Vries, D. J.; Herald, C. L.; Pettit, G. R.; Blumberg, P. M. *Biochem. Pharmacol.* **1988**, *37*, 4069-4073.
13. Wender, P. A.; Cribbs, C. M.; Koehler, K. F.; Sharkey, N. A.; Herald C. L.; Kamano, Y.; Pettit, G. R.; Blumberg, P. M. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7197-7201.
14. Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127-6129.
15. Pettit, G. R.; Sengupta, D.; Blumberg, P. M.; Lewin, N. E.; Schmidt, J. M.; Kraft, A. S. *Anti-Cancer Drug Design* **1992**, *7*, 101-113.
16. a) Mochly-Rosen, D.; Koshland Jr., D. E. *J. Biol. Chem.* **1987**, *262*, 2291-2297. b) Tanaka, Y.; Miyake, R.; Kikkawa, U.; Nishizuka, Y. *J. Biochem.* **1986** *99*, 257-261.
17. Pettit, G. R.; Srirangam, J. K.; Barkoczy, J.; Williams, M. D.; Durkin, K. P. M.; Boyd, M. R.; Bai, R.; Hamel, E.; Schmidt, J. M.; Chapuis, J-C. *Anti-Cancer Drug Design* **1995**, *10*, 529-544.