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TANNINS AS SELECTIVE INHIBITORS OF PROTEIN KINASE C

Yoshiki Kashiwada,[a] Gen-ichiro Nonaka,[b] Itsuo Nishioka,[b] Lawrence M. Ballas,[c] Jack B. Jiang,[c] William P. Janzen,[c] and Kuo-Hsiung Lee[a] *

[a]*Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599*
[b]*Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812, Japan*
[c]*Sphinx Pharmaceuticals Corporation, Durham, North Carolina 27717*

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Abstract: Fifty-six tannins were evaluated for their inhibitory effects against protein kinase C (PKC). Ellagitannins and complex tannins were found to be potent inhibitors of PKC, while gallotannins and condensed tannins, having a relatively large number of phenolic hydroxy groups, showed moderate inhibitory effects on PKC. Phorbol displacement assay suggested that the active tannins interact with the regulatory site of the enzyme.

Protein Kinase C (PKC, Ca^{++}/ Phospholipid-dependent protein kinase) plays an important role in signal transduction as well as various cellular regulations, proliferation, and differentiation.[1] Since a variety of possible roles of PKC in cellular functions have been recognized, the specific inhibitors of PKC might be useful as chemotherapeutic agents for human cancer,[2-4] central nervous system, cardiovascular system, inflammation, immunue system, and other metabolic systems.[1] In the course of our search for potent PKC inhibitors from natural products, we have found that in addition to tannic acid, some Chinese crude drugs, such as rhubarb and *Sanguisorba officinalis* showed strong inhibitory effects against PKC. Further bioassay-directed fractionation led to the isolation of known tannins, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**7**),[5] procyanidins B-2 3,3'-di-*O*-gallate (**50**)[6] and C-1 3,3',3"-tri-*O*-gallate (**51**),[6] and sanguiin H-11 (**41**)[7] from tannic acid, rhubarb, and *S. officinalis*, respectively, as the anti-PKC principles. Since these are the first identified tannins to demonstrate potent anti-PKC activity,[8] we have screened the other tannins as potent anti-PKC agents.

The 56 tannins[9] examined for the PKC-inhibitory effect[10] can be classified into four groups ; gallotannins (**1** - **17**), ellagitannins (**18** - **44**), condensed tannins (**45** - **54**), and complex tannins (**55** and **56**).

As shown in Table 1, pentagalloyl glucose (**7**) was found to be the most potent inhibitor of PKC among the gallotannins. It was shown that at least four galloyl groups are needed for the anti-PKC activity.

A comparison of the activities of **19** - **24** with those of the corresponding galloylglucoses suggested that the hexahydroxydiphenoyl (HHDP) group is more important than the digalloyl moiety in contributing to the enhanced anti-PKC activity, despite the fact that both the HHDP group and the digalloyl moiety possess the same number of phenolic hydroxy groups. The location of the HHDP group seems to be unimportant for this activity since the glucose core-bearing ellagitannins contain the same number of phenolic hydroxy groups and exhibit a similar level of inhibitory activity. Furthermore, increased activity was observed in tcompounds having a large

number of phenolic hydroxy groups. The dimeric and tetrameric ellagitannins, however, showed activity comparable to that of **21** and **22**, which are the monomeric units of **40** - **42**.

The *C*-glycosidic ellagitannins (**35** - **39**) show similar activity, and their activities are similar to that of **21**, suggesting that the *C*-glycosylation is not responsible for the activity.

Compounds **23** and **31**, as well as compounds **25** and **32**, are structurally related. However, they are different in that the HHDP group of **23** and **25** is replaced by a valoeaoyl group. They showed similar activity, although the valoeaoyl group is composed of an HHDP and a galloyl groups.

The dehydrohexahydroxydiphenoyl (DHHDP) and chebuloyl groups are metabolites of the HHDP group. The compounds having these acid groups (**25**, **27** - **30**) were shown to be less active than **23** and **24**.

The comparison of **33** - **35** with **19**, **20** and **39**, respectively, suggests that the activity of the gallagyl group is similar to that of the HHDP group, although the gallagyl group has two HHDP group units.

Compounds **43** and **44**, possessing a triterpenoid moiety and an HHDP group, showed stronger activity than **18**, which has the same number of HHDP groups in the molecule. The triterpenoid moiety might take part in anti-PKC activity.

In the case of condensed tannins, the activity was increased according to the number of phenolic hydroxy groups, similar to that found in other classes of tannins. The galloyl group at flavan C-3 was shown to enhance the activity.

Table 1. Anti-PKC Activities for Tannins

Compd. No.	Anti-PKC activity $IC_{50}(\mu M)$	Phorbol Displacement	c-AMP kinase $IC_{50}(\mu M)$	Compd. No.	Anti-PKC activity $IC_{50}(\mu M)$	Phorbol Displacement	c-AMP kinase $IC_{50}(\mu M)$
1	>100	NT	NT	**29**	30	NT	NT
2	>100	NT	NT	**30**	30	NT	NT
3	>100	NT	NT	**31**	20	+	100
4	>100	NT	NT	**32**	16	+	65
5	20	+	-	**33**	10	NT	NT
6	82	+	-	**34**	8	NT	NT
7	4	+	NT	**35**	4	NT	NT
8	> 100	NT	NT	**36**	4	+	-
9	>100	NT	-	**37**	8	+	-
10	>100	+	-	**38**	12	+	-
11	>100	NT	NT	**39**	11	+	84
12	64	+	-	**40**	3	+	32
13	>100	NT	NT	**41**	5	+	48
14	>100	NT	NT	**42**	10	+	22
15	>100	NT	NT	**43**	18	+	-
16	>100	NT	NT	**44**	23	+	-
17	>100	NT	NT	**45**	>100	NT	NT
18	>100	NT	NT	**46**	>100	NT	NT
19	15	NT	-	**47**	>100	NT	NT
20	4	+	-	**48**	40	+	-
21	3	+	-	**49**	48	+	-
22	11	+	-	**50**	4	+	-
23	20	NT	NT	**51**	2	+	-
24	4	+	-	**52**	>100	NT	NT
25	11	NT	-	**53**	30	+	-
26	17	+	-	**54**	15	+	-
27	86	NT	NT	**55**	7	+	95
28	16	+	-	**56**	4	+	44

NT : Not tested
Phorbol displacement assay + : did displace phorbol (PDBu) at 50 μM or less
c-AMP kinase assay - : did not inhibit c-AMP kinase

The activity of complex tannins was greater than that of their component units (**37** and **45**).

The phorbol displacement assay[11] was carried out with the thirty selected tannins for their mechanism of action study. All of the tannins tested did displace phorbol, suggesting that the active tannins interact with the regulatory site of the enzyme. Furthermore, most tannins were found to show no inhibitory activity against c-AMP-dependent protein kinase,[12] although **31, 32, 39 - 42, 55,** and **56** showed a small degree of inhibition against this enzyme. This result indicated the anti-PKC activity of these tannins is selective.

Studies on the intracellular and/or *in vivo* effects of those active compounds is in progress.

References and Notes

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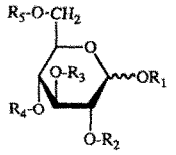
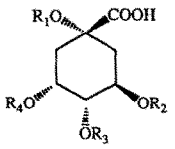
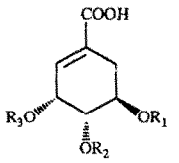
10. Protein Kinase C Assay : Small unilamellar vesicles consisting of 40 μg/ml phosphatidylserine (Avanti) and 1.76 μg/ml diacylglycerol (Avanti) in 20 mM HEPES buffer (pH = 7.5, Sigma), 10 mM $MgCl_2$ (Sigma), 200 μg/ml histone (type HL, Worthington), 100 μM $CaCl_2$ (Sigma), 47.5 μM EGTA (Sigma), and 20 μM ^{32}P-APT (DuPont). The assay is started by addition of PKC, incubated at 30 °C for 10 minutes, and stopped by adding 0.5 ml ice cold trichloroacetic acid (Amresco) followed by 100 μl of 1 mg/ml Bovine serum albumin (Sigma). The precipitate is collected by vacuum filtration on GFC filters and quantified by counting in a beta scintillation counter.
11. Phorbol Displacement Assay: Vesicles of 40 μg/ml phosphatidylserine (Avanti) in 20 mM Tris-HCl, 2 mM $CaCl_2$ (Sigma), and 100 nM [^{3}H]PDBu (DuPont), and tannins (11 or 50 μM). Nonspecific binding of [^{3}H]PDBu is measured in a duplicate tube containing 20 μM PDBu (Sigma). The reaction is initiated by addition of PKC, incubated at room temperature for 30 minutes, and then refrigerated. Bound material is collected by filtration on GFC filters and washing with an ice cold buffer consisting of 5 mM Tris-HCl and 0.2 mM $CaCl_2$. The bound material is then counted in a beta scintillation counter.
12. c-AMP Dependent Protein Kinase Assay : Assay components are : 20 mM HEPES buffer (Sigma), 200 μg/ml histone type HL (Worthington), 10 mM $MgCl_2$ (Sigma), and 20 μM (^{32}P-τs)ATP (DuPont). Assays are performed plus and minus 0.3% Triton X-100 (Amresco). The procedure was similar to that of ref. 10 except for using bovine heart c-AMP-depentant kinase catalytic subunit (Sigma) insteatd of PKC.



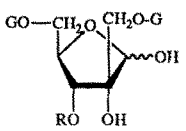
	R_1	R_2	R_3	R_4	R_5
1	G(β)	H	H	H	H
2	G(β)	H	H	H	G
3	G(β)	G	H	H	G
4	G(β)	H	H	G	G
5	G(β)	G	G	H	G
6	H	G	G	G	G
7	G(β)	G	G	G	G



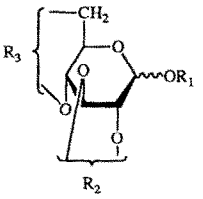
	R_1	R_2	R_3	R_4
8	H	H	G	H
9	G	H	G	H
10	G	H	G	G
11	H	G	G	G
12	G	G	G	G



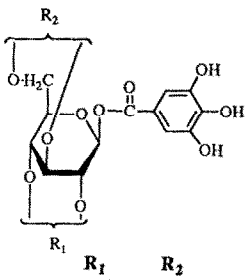
	R_1	R_2	R_3
13	G	H	H
14	G-G	H	H
15	G	H	G



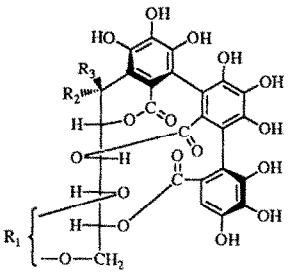
	R
16	H
17	G



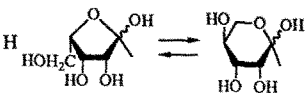
	R_1	R_2	R_3
18	H	*S*-HHDP	H, H
19	G(β)	H, H	*S*-HHDP
20	H	*S*-HHDP	*S*-HHDP
21	G(β)	*S*-HHDP	*S*-HHDP
22	G(β)	G, G	*S*-HHDP
33	H	H, H	Gal
34	H	*S*-HHDP	Gal



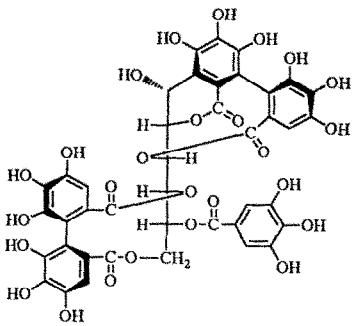
	R_1	R_2
23	H, H	*R*-HHDP
24	G, G	*R*-HHDP
25	DHHDP	*R*-HHDP
26	Ela	*R*-HHDP
27	DHHDP	H, H
28	DHHDP	G, G
29	Che	G, G
30	Che	*R*-HHDP
31	H, H	Val
32	DHHDP	Val



	R_1	R_2	R_3
35	Gal	OH	H
36	*S*-HHDP	H	OH
37	*S*-HHDP	OH	H



38	*S*-HHDP	H



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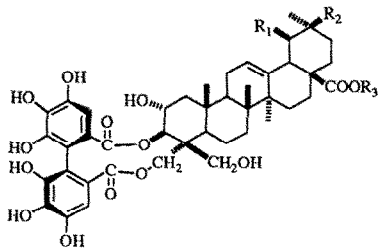
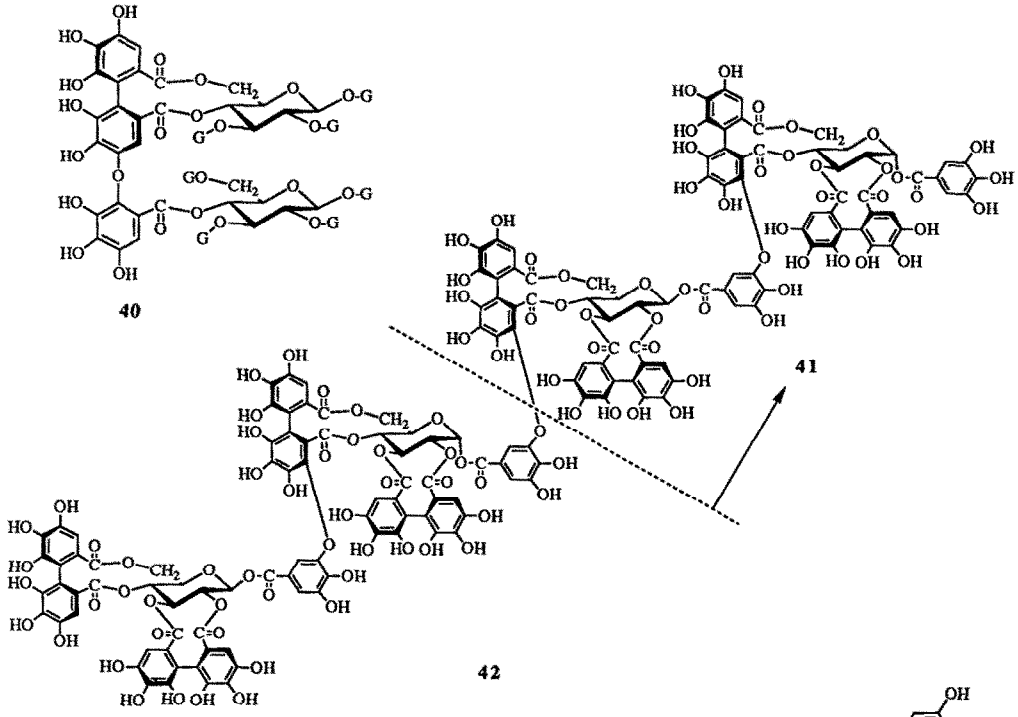


	R_1, R_2	R_3
43	H, CH_3	β-D-Glc
44	H, CH_3	H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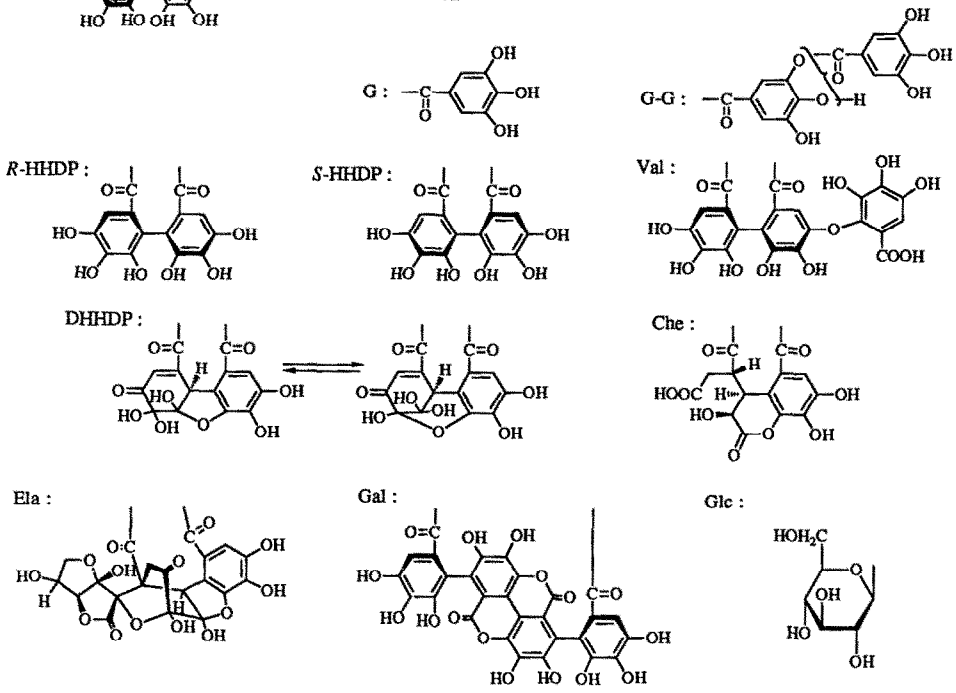
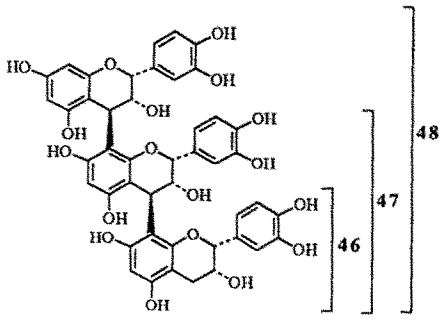
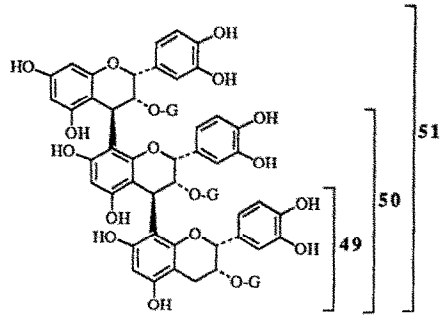
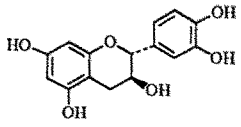

G :
G-G :
R-HHDP :
S-HHDP :
Val :
DHHDP :
Che :
Ela :
Gal :
Glc :







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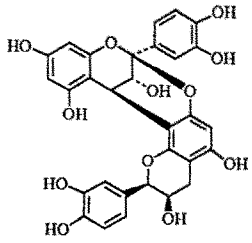

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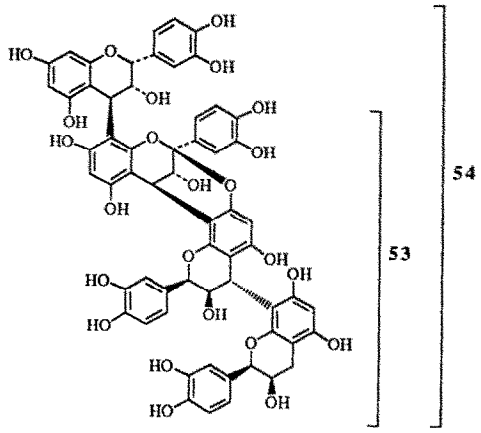

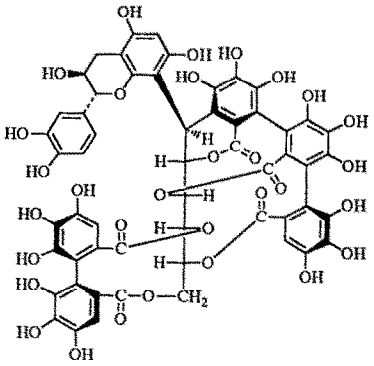

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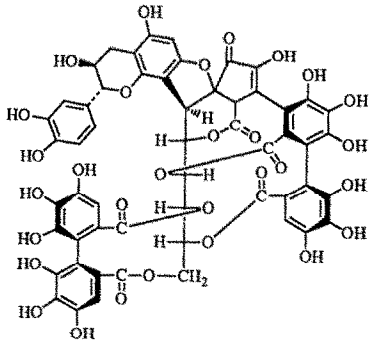

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