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THE CONSERVED ACID BINDING DOMAIN MODEL OF INHIBITORS OF PROTEIN PHOSPHATASES 1 AND 2A: MOLECULAR MODELLING ASPECTS.

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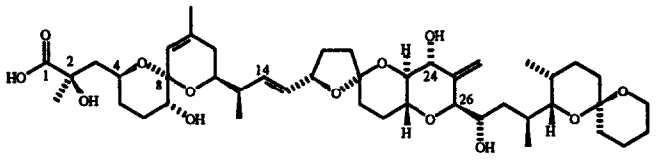
Abstract: Using molecular modelling, three chemically distinct members of the okadaic acid class of protein phosphatase inhibitors and tumor promoters, okadaic acid, calyculin A and microcystin-LR were fitted together. The molecular modelling results indicate a pharmacophore model consisting of a central core, containing one conserved acidic group and two potential hydrogen bonding sites, and a non-polar side chain

The binding of the okadaic acid class of compounds to their receptors, the catalytic subunits of protein phosphatases 1 and 2A, causes an inhibition of phosphatase activity resulting in modulation of cellular events controlled by phosphorylation/dephosphorylation mechanisms.[1-6] A general biochemical pathway of tumor promotion, the okadaic acid pathway, which is mediated through inhibition of protein phosphatases 1 and 2A has been shown to occur.[7] Okadaic acid (1)[8], calyculin A (2)[9], and microcystin-LR (3)[10] are representative of the structurally diverse group of compounds comprising the okadaic acid class. Inhibition of protein phosphatases by okadaic acid results in increased protein phosphorylation *in vitro* and in cells.[3b,11] The IC_{50} values (nM) for inhibition of the purified catalytic subunits of PP1 and PP2A by the okadaic acid class indicate



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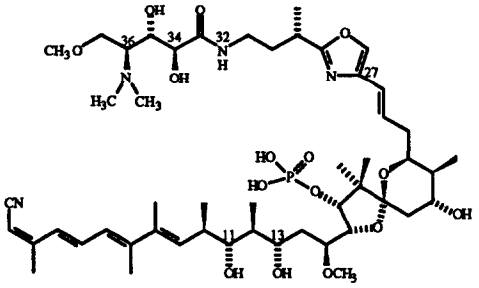

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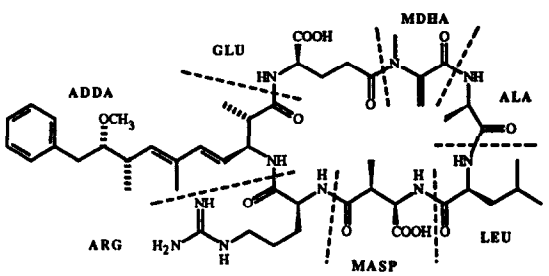

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that all the compounds are potent inhibitors and have similar activity (Table 1).[12] It was shown that the three compounds bind to the same site of the protein phosphatases as they inhibit specific [^{3}H]okadaic acid binding (Table 1).[5,7] Since the three structurally unrelated compounds interact at the same modulatory site of protein phosphatases they would be expected to possess a common pharmacophore.

Table 1. Inhibition of the Protein Phosphatases 1 and 2A by the Okadaic Acid Class Compounds.

	Inhibition of the purified catalytic subunit of PP1 from rabbit skeletal muscle, IC_{50} (nM) [12]	Inhibition of the purified catalytic subunit of PP2A from human erythrocytes, IC_{50} (nM) [12]	Inhibition of specific [^{3}H]okadaic acid binding to the cytosolic fraction of mouse liver [5a] or mouse skin[7], ED_{50} (nM)
Okadaic acid	3.4	0.07	2.8 [7], 3.2 [5a]
Calyculin A	0.3	0.13	2.8 [7]
Microcystin-LR	0.1	0.10	1.3 [5a]

A pharmacophore model of the okadaic acid class compounds was developed using computer assisted molecular modelling. The computer generated minimum energy conformations of okadaic acid and calyculin A are shown in Fig. 2.[13] Okadaic acid was found to assume a cyclic conformation forming a cavity held together by an intramolecular hydrogen bond between the C1 carboxyl, attached to the first tetrahydropyran ring and the C24 hydroxyl group attached on the fourth tetrahydropyran ring. The two terminal tetrahydropyran rings were separated from the cavity region by an extended alkyl chain attached to C26. There was a second intramolecular hydrogen bond between the C2 hydroxyl and the ether oxygen O^4 joining C4 and C8. These intramolecular hydrogen bonds were also observed in the x-ray crystal structure of acanthifolicin[14] a structurally related compound, and the *o*-bromobenzyl ester derivative of okadaic acid.[8] The crystal structure of calyculin A shows there is hydrogen bonding within the molecule forming a cavity similar to that formed in okadaic acid.[9] The phosphoric acid oxygens are hydrogen bonded to the C13 hydroxyl group, the tertiary amine N^{36} and the oxazole nitrogen N^{27}. The C11 hydroxyl group hydrogen bonds to the amide nitrogen N^{32}.

Fitting of the conformations was pursued in order to identify common structural features which would explain the binding of the ligands to the same protein receptor. Receptor binding involves steric, hydrophobic and electrostatic interactions of the ligands with the protein and pattern recognition plays an important part of our approach to identifying the common features among ligands.[15] Structure-activity studies have indicated that a carboxylic acid functionality is essential for okadaic acid to bind to its receptor as the methyl ester derivative showed no inhibition of specific [^{3}H]okadaic acid binding and no inhibition of protein phosphatase activity.[16] As there is a phosphoric acid group in calyculin A and two carboxylic acid groups in microcystin-LR, initial studies were undertaken to determine if this common feature was significant. The compounds with one acidic group, okadaic acid and calyculin A, were viewed along the C1=O and P=O bonds, respectively, in order to identify any similarities between the two compounds. This mimicked the features the enzyme may recognize if the acids were bound to the same amino acid residue. Simple pattern recognition lead to the observation that the cavities of each compound occupied a similar region and that the side chains extended into a second common domain. From these views, it appeared possible to achieve an alignment of the two molecules in which the two side chains occupied the same region. Closer examination revealed that within this pattern of similarity there were two other alignments; (I) the tetrahydropyran ether oxygen O^8 of okadaic acid and the oxazole nitrogen N^{27} of calyculin A and (II) the hydroxyl oxygen O^{24} of okadaic acid and the hydroxyl oxygen O^{34} of calyculin A. Rigid superimposition of the okadaic acid and calyculin A was carried out using the alignment of these oxygens and the nitrogen. This resulted in superimposition of the two cavities and the two side chains; the ketal side chain of okadaic acid and the nitrile side chain of calyculin A (Fig. 2C).

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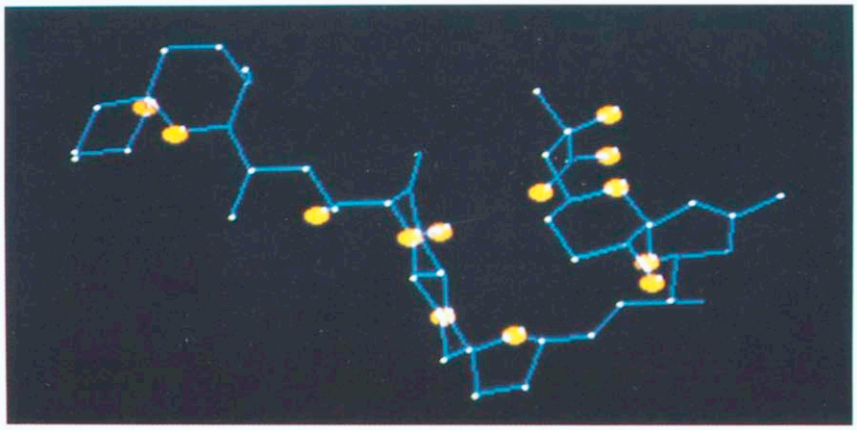

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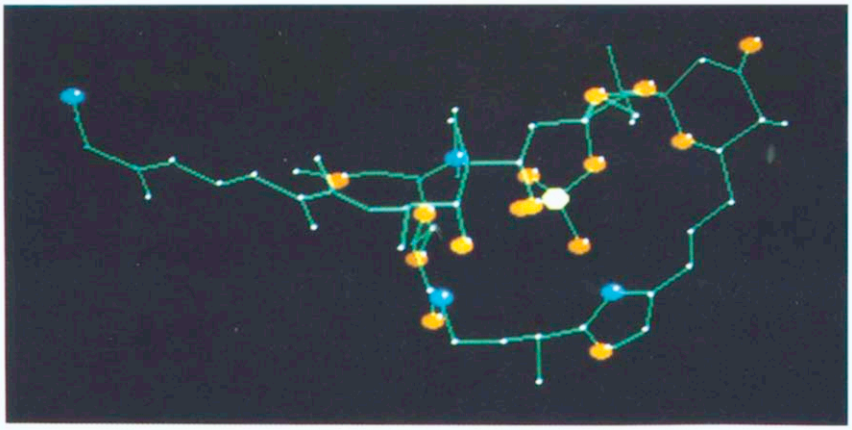

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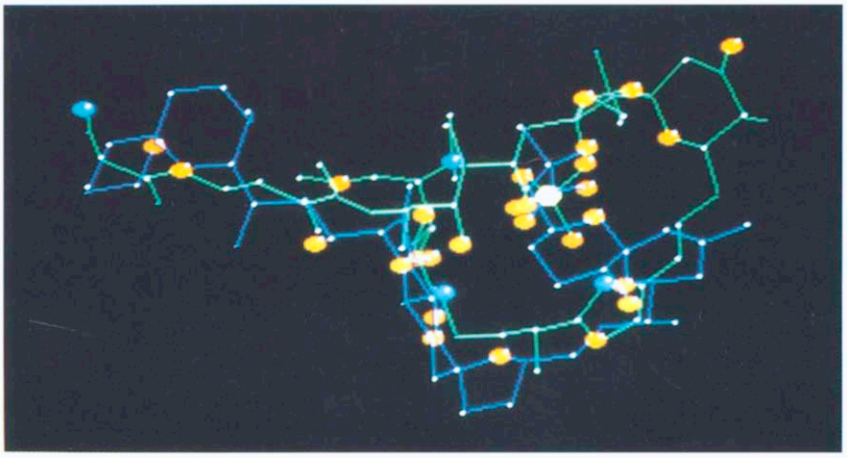
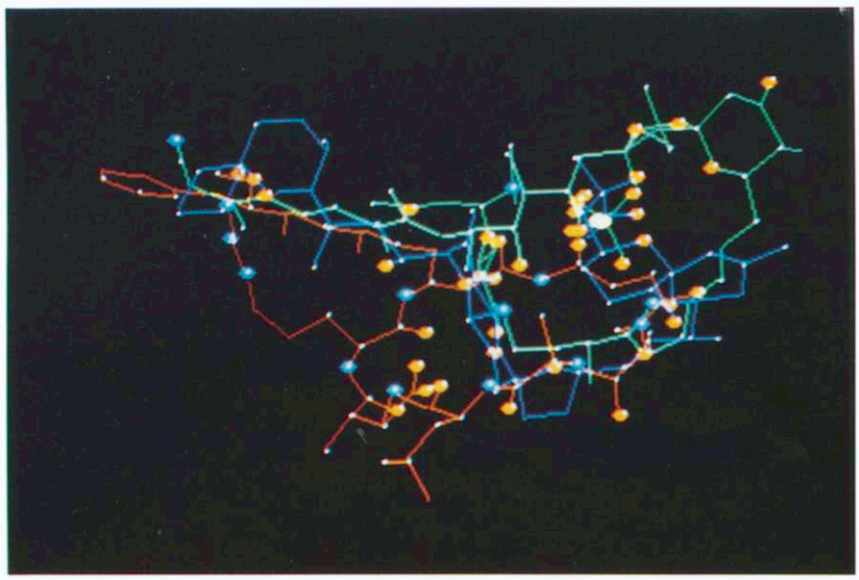


Figure 2. (A) Minimum energy conformation of okadaic acid, (B) Minimum energy conformation of calyculin A, (C) Superimposition of okadaic acid and calyculin A.



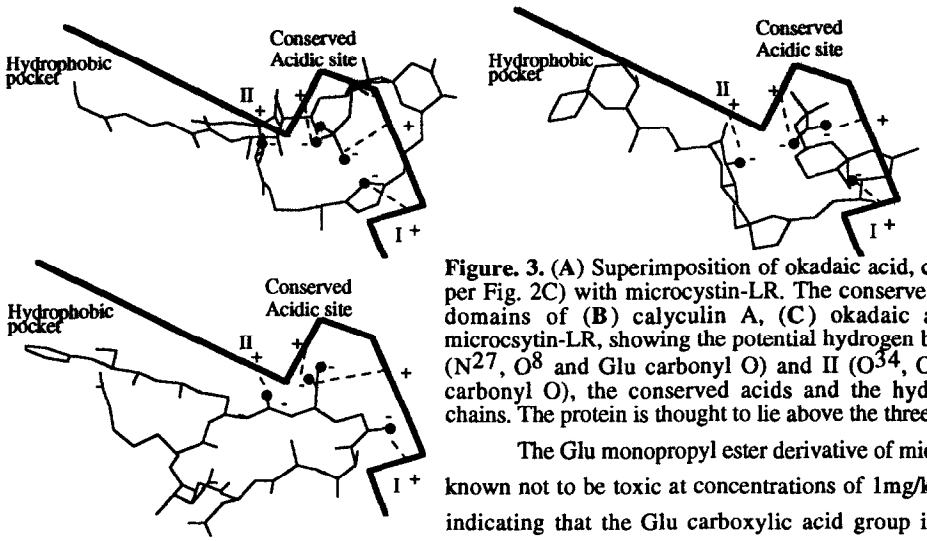


Figure. 3. (**A**) Superimposition of okadaic acid, calyculin A (as per Fig. 2C) with microcystin-LR. The conserved acid binding domains of (**B**) calyculin A, (**C**) okadaic acid and (**D**) microcsytin-LR, showing the potential hydrogen bonding areas I (N^{27}, O^{8} and Glu carbonyl O) and II (O^{34}, O^{24} and Adda carbonyl O), the conserved acids and the hydrophobic side chains. The protein is thought to lie above the three compounds.

The Glu monopropyl ester derivative of microcystin-LR is known not to be toxic at concentrations of 1mg/kg (ip, mouse), indicating that the Glu carboxylic acid group is essential for toxicity, probably mediated via inhibition of protein phosphatases.[17] The computer generated minimum energy conformation of microcystin-LR has been reported.[18] Microcystin-LR was viewed along the C=O bond of the Glu and compared to the superimposition of okadaic acid and calyculin A viewed in a similar fashion as discussed above. There was alignment with both the previously identified potential hydrogen bonding groups, such that the Glu carbonyl O of the peptide bond aligned with area I and the Adda carbonyl O of the peptide bond aligned with area II. Rigid fitting using the alignment of the hydrogen bonding groups in microcystin-LR with the corresponding sites in okadaic acid and calyculin A was undertaken. The cyclic peptide ring of

microcystin-LR aligned with the common cavities of okadaic acid and calyculin A and the Adda side chain superimposed with the ketal side chain of okadaic acid and the nitrile side chain of calyculin A (Fig. 3A).

The molecular modelling results indicated that the pharmacophore model required a central core consisting of one conserved acidic group and two potential hydrogen bonding sites (I and II), and a non-polar side chain (Fig. 3B, C and D). The pharmacophore model of okadaic acid, calyculin A and microcystin-LR highlight the conserved acidic group. This model is consistent with the rather limited structure-activity information currently known of the okadaic acid class of protein phosphatase inhibitors. The conserved nature of the acid is evident by the significant decrease in the inhibition of specific [^{3}H]okadaic acid binding by the methyl ester of okadaic acid, the methyl ester of acanthifolicin, okadylamine, okadanol, nor-okadanone, nor-okadanol and the monopropyl ester of microcystin-LR.[16,17] Okadaic acid tetramethyl ether was also found to be inactive, this finding supports the proposal that area I and area II, the potential hydrogen bonding sites within the cavity, are necessary. The hydrophobic side chain of the pharmacophore model extends from the central core away from the conserved acidic site. As the side chain region contains a ketal (okadaic acid), a nitrile (calyculin A) and an olefin (microcystin-LR) the potential interactions with receptor proteins might be expected to be similar. When the configuration of Adda in microcystin-LR is altered to 4(E),6(Z), binding is diminished indicating that this region is required for receptor binding. A minor component having the 4(E),6(Z) stereochemistry was found to bind to the okadaic acid receptors in the cytosolic fraction of mouse skin with an ED_{50} of 68 nM compared to microcystin-LR which had an ED_{50} of 8 nM.[19] In a similar manner, the E configuration about the C14-C15 double bond of okadaic acid has been shown to be necessary for activity.[20] Replacement of the arginine in microcystin-LR with alanine does not destroy binding to the receptor site.[21] This is verified by the recent isolation of a second cyclic pentapeptide, motuporin, found to inhibit protein phosphatase 1 at <1 nM.[22] Motuporin is similar in structure to nodularin, but with the arginine residue replaced by a valine residue. Modification of the variant region of the microcystins does not alter the toxicity while microcystin-LR, -YR and -RR have the same activity for inhibition of protein phosphatases 1 and 2A and inhibition of ^{3}H-okadaic acid binding [5a]. The cavity caused by intramolecular hydrogen bonding in okadaic acid and calyculin A results in these compounds being similar in shape to the cyclic peptide microcystin-LR with the intramolecular hydrogen bonding being necessary to hold the correct conformation to allow interaction at the receptor site of the protein phosphatases.

Molecular modelling has allowed common regions of okadaic acid, calyculin A and microcystin-LR to be recognized and a pharmacophore model developed. The pharmacophore model consists of a central core, containing one conserved acidic group and two potential hydrogen bonding sites (I and II), and a non-polar side chain (Fig. 3B).

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Computer graphic analyses were performed on a VAX 11/750 computer and a Macintosh IIsi terminal with CHEM-X (Jan 90) molecular modelling software using the CDL GKS 3D graphics window and on a micro VAX 3600 with CHEM-X (July 91). The crystal structure of the *o*-bromobenzyl ester of okadaic acid [8] was retrieved from the Cambridge Structural Database (*Cambridge Crystallographic Data Centre, Lensfield Rd, Cambridge, U.K.*). This was the starting point for the generation of the global minimum energy conformations of okadaic acid (Fig. 2A). The minimum energy conformation was obtained by performing a systematic conformational search about rotatable bonds at increments of 30°. From this series of conformations (12^{10} conformations) the minimum energy conformation was selected and the global minimum was determined using van der Waals energy (VDWE) calculations and Molecular Mechanics optimizations (MME). The VDWE calculations minimise the conformation by maximising the non-bonded interactions between atoms. The MME option minimises the energy by optimizing the bond lengths and bond angles of the conformation. The crystal structure of calyculin A was retrieved from the CSD.[9] The absolute stereochemistry of calyculin A was been reported to be the enantiomer of the x-ray crystal structure in the CSD.[23] The correct enantiomer was calculated by translating the x-coordinates of the original North file and transferring into CHEM-X. VDWE calculations were performed to obtain the global minimum energy. Microcystin-LR was constructed from constituent amino acids using the CHEM-X 'modification and building' option.[10,18] The CHEM-X (Jul 91) programme and the distance geometry interface, DGEOM, were used in order to generate the global minimum energy. DGEOM takes into account the explicit connectivity of the initial structure and is able to locate the non-rotatable bonds and set restraints according to the bond type, such as the amide bond.[24] Conformations are then calculated randomly to give the lowest energy conformation. The lowest energy conformation was then subjected to VDWE calculations and to MME to generate the global minimum energy conformation. The compounds were superimposed using the 'rigid fit' option to minimize root mean square distances between pairs of selected atoms. Selected pairs of atoms were superimposed without altering the conformations of each compound.

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