# Multivalent Binding in the Design of Bioactive Compounds

Dennis Wright* and Lynn Usher

*328 Chemistry Research Building, Department of Chemistry, University of Florida, Gainesville, FL 32601, USA*

**Abstract:** Over the past several years, much research has centered on the development of small molecules that, through multivalent binding interactions, enhance the regulation of fundamental biological functions. The design of a wide range of potential therapeutics focuses on mimicking natural systems that control the biological effect of protein-ligand interactions through multivalency. In this review, the various approaches to achieving multivalency are discussed. Recent advances in the design of multivalent ligands are also presented.

## INTRODUCTION

The rational design of new therapeutic entities is always advanced by an increase in our understanding of the fundamental biological mechanisms responsible for modulation of potency and affinity. Over the past several years, biochemical studies have shown that the role of multivalent interactions is fundamental to the regulation of many critical biological systems. The unique properties imparted through multivalent binding interactions have inspired a variety of new designs for small molecules that can modulate biological functions. Multivalent interactions, as defined in this review, will involve cases where the ligand has two exclusive binding domains that can simultaneously dock to two or more distinct sites either on the same receptor or on two distinct receptors (Fig. (**1**)).

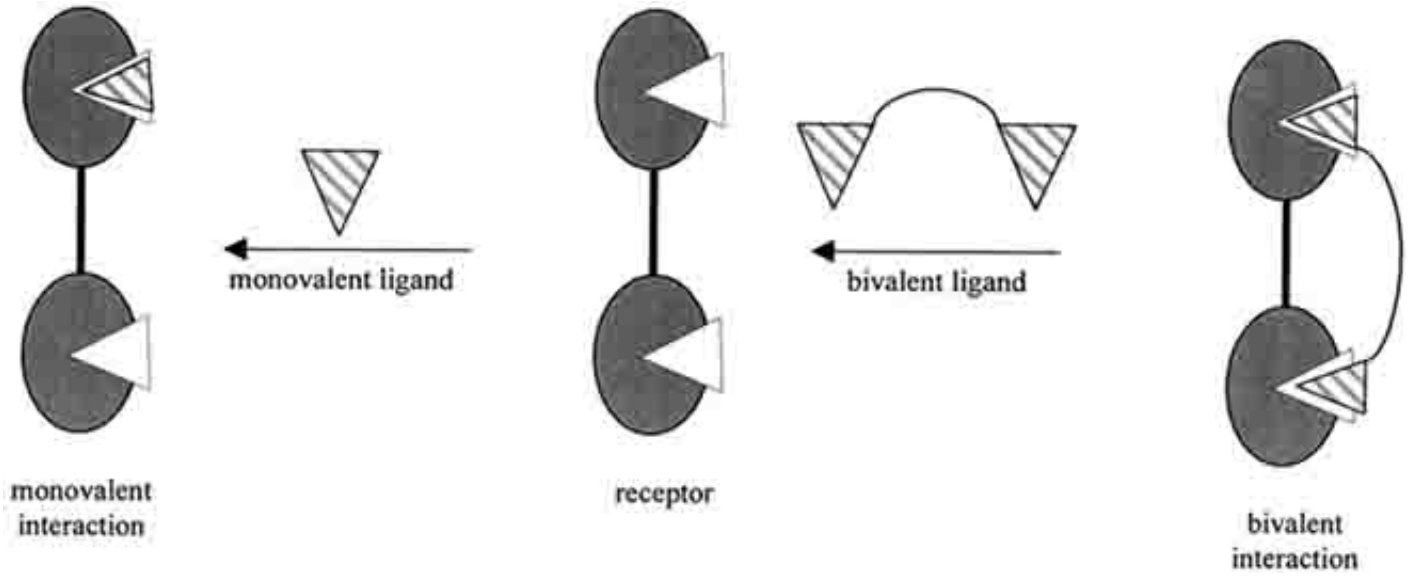


**Fig. (1)**. Monovalent and bivalent interactions.

Many systems in nature rely on the properties of multivalency as a way to modulate the biological effect of protein-ligand interactions. In the past several years, these types of interactions have been exploited in the design of potential drug molecules. One of the reasons multiple binding interactions have evolved in nature is to increase the overall strength of interactions between a ligand and its receptor. This can be an important mechanism to enhance the overall binding strength of a relatively low affinity ligand. Oligomers constructed of repeating units of low affinity ligands that can bind to a receptor containing multiple adjacent receptor sites can yield an overall strong interaction through an increase in avidity. These multiple interactions impart an overall higher avidity for the interaction of a weakly bound ligand and its receptor without increasing the affinity of the individual interactions (Fig. (**2**)).

Perhaps the most well known of these effects are those that mediate the process of cellular adhesion where the interactions are driven primarily by high avidity imparted by

*Address correspondence to this author at the 328 Chemistry Research Building, Department of Chemistry, University of Florida, Gainesville, FL 32601, USA; Ph.: +325-392-6787; Fax: +352-846-0296; e-mail: dwright@chem.ufl.edu

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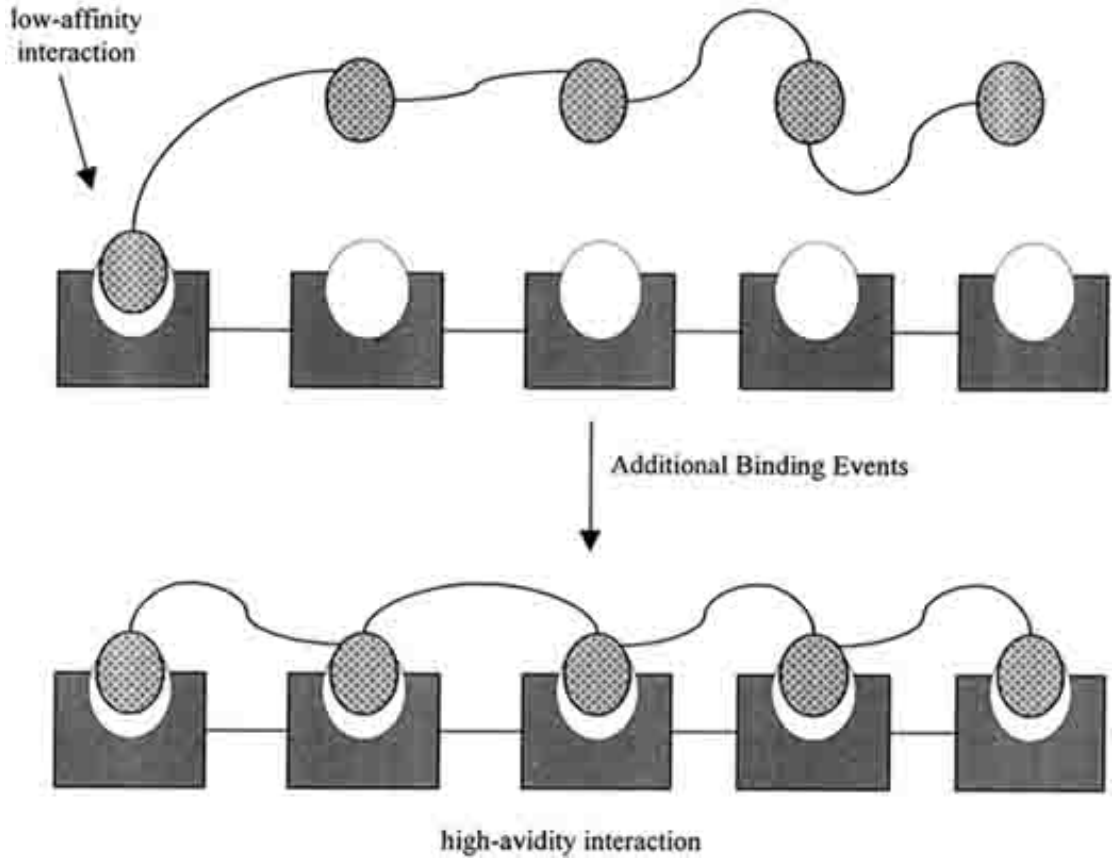



**Fig. (2)**. High avidity interaction.

the use of multiple binding events [1]. The thermodynamic profile of polyvalent interactions has been extensively treated in an excellent review by Whitesides and co-workers [2].

A second type of multivalent interaction can occur when a ligand contains two separate binding domains that dock to two different sites on the same receptor. This type of interaction can increase the overall affinity since the equilibrium between association and dissociation of a ligand from one site on the receptor is greatly influenced by the second binding event at an adjacent site.

Molecules constructed with a second binding site would be expected to show an increase in affinity for the particular receptor or possibly an increase in selectivity since the second interaction may allow the ligand to discriminate between two structurally related receptors.

A final classification for multivalent interactions can occur when a bivalent ligand can bind two different biomacromolecules, such as proteins, and bring them into contact [3]. This induced proximity mechanism can lead to cross-linking or clustering of receptors which has been

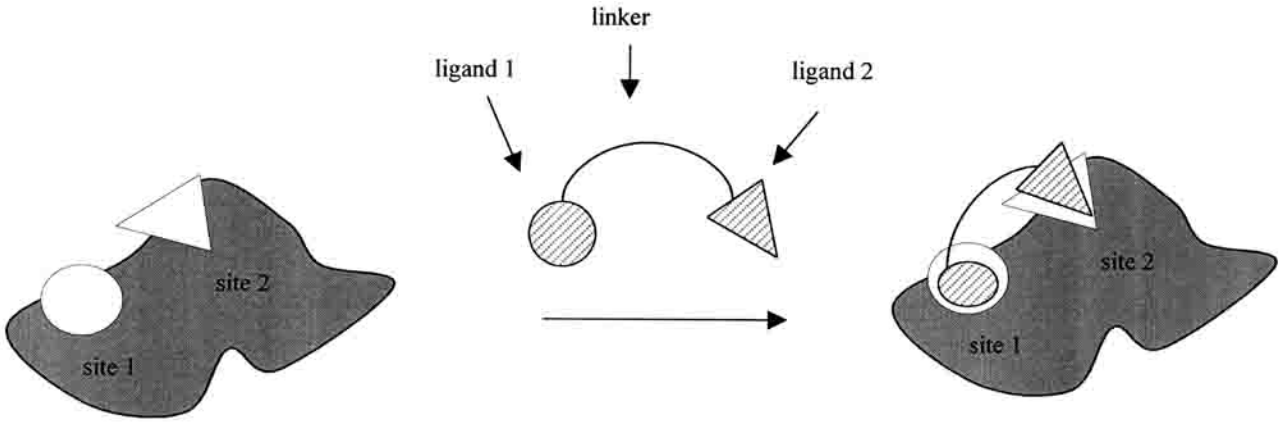



**Fig. (3)**. High affinity binding through bivalent interaction.

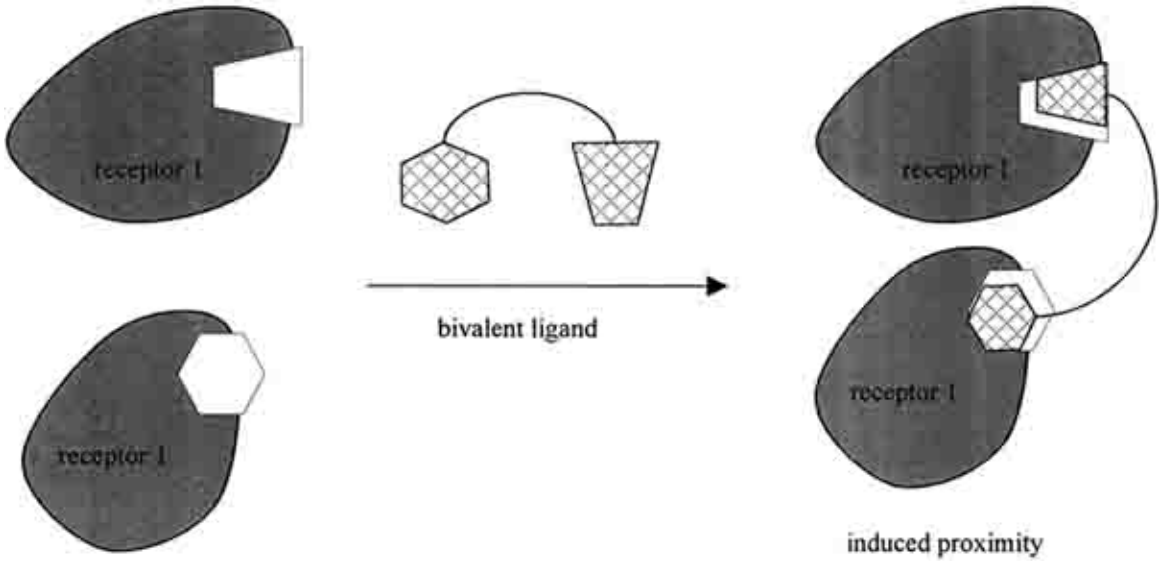



**Fig. (4)**. Bivalent ligand promotes receptor dimerization.

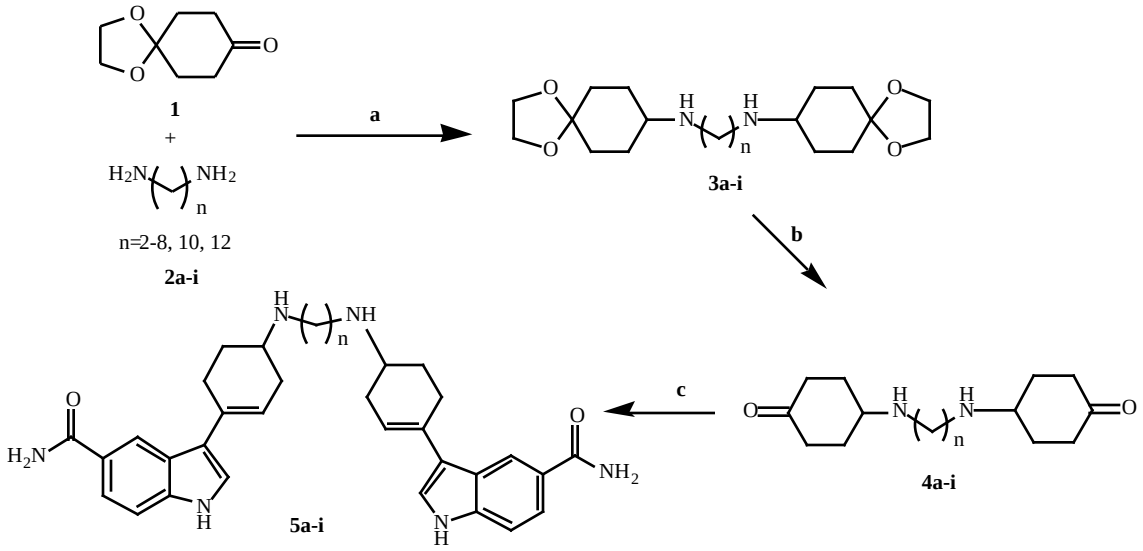
demonstrated to be a key mechanism involved in a variety of signaling cascades (Fig. (**4**)).

This type of interaction has been observed in a variety of biological systems such as the cross-linking of the receptor tyrosine kinase Trk A by nerve growth factor (NGF) which is present as a circulating homodimer [4]. The induced proximity of the two membrane bound receptors lead to phosphorylation of one kinase by the other and ultimately results in propagation of the signal induced by NGF. Several other growth factor based signaling systems have been studied and this type of interaction would appear to be quite general.

Many studies relating to the molecular mechanisms of small-molecule/large-molecule interactions have shown that the use of two or more separate binding events can be used to control biological interactions. These studies have prompted a number of researchers to investigate this mode of action for the design of new therapeutic agents. Several molecules have been designed, synthesized and tested to take advantage of multivalent interactions to enhance affinity, increase avidity or induce proximity. This review will focus on small molecules that have been designed to capitalize on these effects to enhance the overall performance of a drug including issues of potency and selectivity.

## INVESTIGATION OF MULTIVALENT LIGANDS

An interesting study by LeBoullec and co-workers demonstrated the effectiveness of ligand homodimerization in increasing the selectivity and potency of a series of indole derivatives for certain members of the 5HT family of seretonergic receptor subtypes, $5HT_{1A}$ and $5HT_{1D}$ [5]. LeBoullec *et al.* showed that variations in the length of the




**a)** $NaBH(OAc)_3$/ $CH_2Cl_2$/ RT/ 18 hrs; **b)** 1:1 Solution of 50% $H_2SO_4$ - THF/ RT/ 18 hrs; **c)** 5-Carboxamidoindole/ EtOH/ pyrrolidine/ Reflux/ 3 days

**Scheme 1**. Synthesis of bivalent indoles.

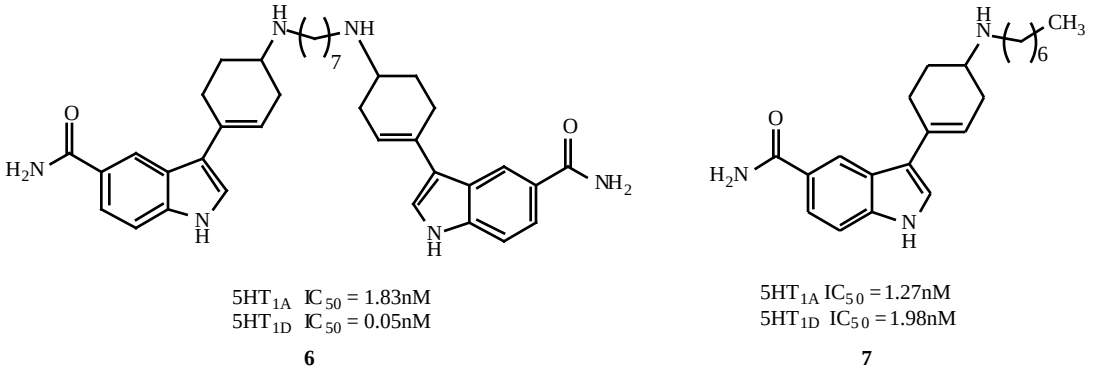
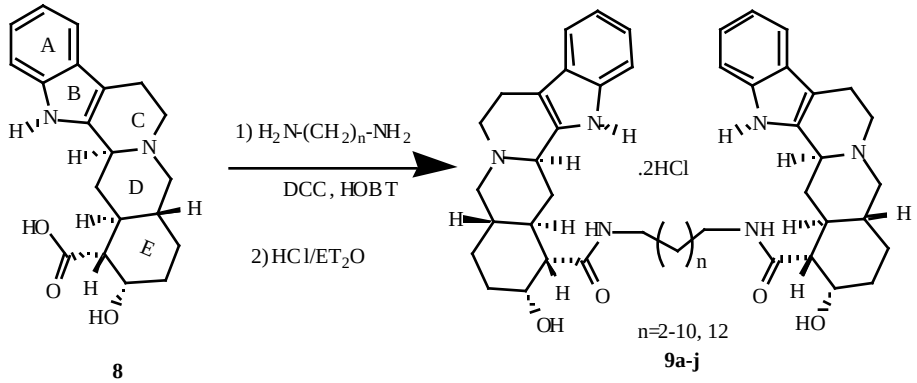



**Fig. (5)**. Bivalent indole 6 binds with 40 fold greater affinity for the $5HT_{1D}$ receptor than monomer **7**.

linker between the two monomers was sufficient to modulate both the binding affinity and receptor specificity of the homodimeric ligands. A series of bivalent indoles were synthesized as shown in Scheme 1. Double reductive amination of linear diamines with the monoketal of cyclohexane-1,4-dione delivered bis-ketals **3.** Deprotection to the diketone was followed by addition of the 5-substituted indoles and dehydration to deliver the bis-indole derivatives **5**.

The 5-carboxamidoindoles having chain lengths of seven and eight methylene units were selective for the $5HT_{1D}$ subtype and possessed the greatest potency for the receptor, having $IC_{50}$ values of 0.05nM and 0.11nM, respectively. Furthermore, selectivity for the $5HT_{1D}$ site over the $5HT_{1A}$ was dramatically increased by a factor of 39 and 19 respectively as compared to the indole monomer **7** (Fig. (**5**)). Analogs with longer linkers exhibited no selectivity for the $5HT_{1D}$ receptor over the $5HT_{1A}$ receptor.

Another study exploring ligand homodimerization as a strategy to increase receptor selectivity has been reported by Miller *et al*. through the synthesis of yohimbine dimers [6]. Miller and colleagues used the concept of ligand bivalency to identify molecules capable of distinguishing between α-adrenoreceptor subtypes. A series of yohimbine dimers were synthesized as outlined in Scheme 2 by direct coupling of a diamine to the E ring carboxyl group found in yohimbinic acid (**8**).




**Scheme 2**. Synthesis of yohimbine dimers.

The parent molecule, yohimbine (**10**) (Fig. (**6**)), is a potent and selective $\alpha_2$-AR antagonist; however, it is unable to distinguish between the three $\alpha_2$-AR subtypes, hence the interest in dimerization as a means of increasing subtype

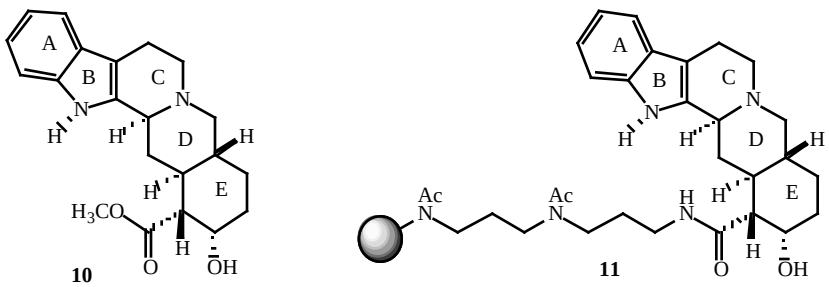
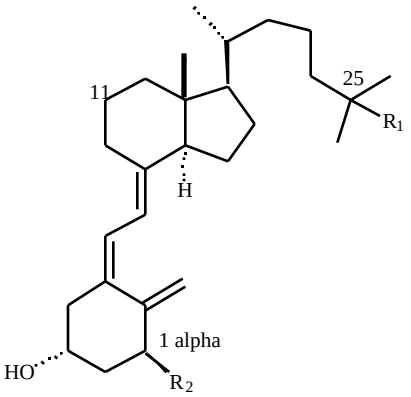



**Fig. (6).** Yohimbine (**10**) and the yoimbine-agarose conjugate (**11**).




**12a**, $R_1 = R_2 = H$; vitamin $D_3$
**12b**, $R_1 = OH$, $R_2 = H$; 25-OH-$D_3$
**12c**, $R_1 = R_2 = OH$; 1alpha, 25-$(OH)_2$-$D_3$

**Fig. (7)**. Vitamin $D_3$ (**12a**) and calcitrol (**12c**).

selectivity. The C-16 carboxyl of yohimbine was chosen as the site of attachment for the linker based on previous reports that the yohimbine-agarose conjugate **11** (Fig. (**6**)) actively binds to the $\alpha_2$-AR substrates, indicating that derivitization of C16 does not interfere with binding.

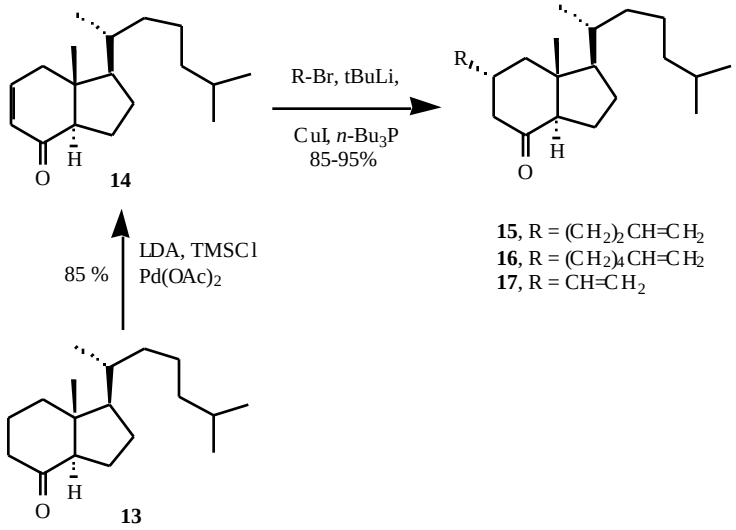
The authors report that although dimerization did not increase affinity for the receptor, possibly due to linker length, the lack of enhanced affinity was more pronounced for the $\alpha_{2b}$AR than for the $\alpha_{2a}$AR, indicating a mechanism of selectivity related to the structure of the receptor subtypes. Miller *et al*. propose that the presence of a highly positively charged extracellular loop on the $\alpha_{2b}$AR (absent in the $\alpha_{2A}$ subtype) results in strong electronic repulsions with one moiety of the yohimbine dimer, destabilizing the complex and therefore decreasing affinity. Since this destabilizing effect is not a factor with the $\alpha_{2a}$ subtype, the yohimbine dimers bind more favorably to this receptor subtype. Of the dimers studied, those with linkers of five, six, eight, and ten carbons showed greatest selectivity (48-fold to 123-fold). Compound **9e** where n=6 showed peak selectivity ($\alpha_{2a}/\alpha_{2b}$ =123).

Sarandeses and colleagues extended this use of multivalency to Vitamin D receptor homo- or heterodimerization [7]. Vitamin $D_3$ (**12a**) (Fig. (**7**)) in its active form, calcitrol (**12c**) (Fig. (**7**)), regulates a broad range of biological processes including calcium and phosphorous metabolism, cellular differentiation, and proliferation of tumor cells. Through the synthesis of vitamin $D_3$ and calcitrol dimers linked at the C11 position, Sarandeses *et al*. propose the possibility of controlling the degree of induction of vitamin $D_3$ protein receptor dimerization.

The synthetic route used to construct the dimers is based on SAR that indicated that the hydroxy groups at C3, C1α and C25 are essential for Vitamin $D_3$ binding and therefore must remain unaffected upon conjugation to a linker to form the dimer. SAR studies also indicated that the introduction of any polar groups on the triene system drastically alters biological activity. The synthetic routes outlined in Schemes 3 and 4 allowed the preparation of linkers of variable length and rigidity so that the optimal structure for VDR binding could be determined.

The synthetic strategy relies on dimerization of a key intermediate prior to installation of the somewhat sensitive triene unit. Conjugate addition of an alkenyl tether to a CD hydrindane system **14** introduces a functional handle for dimerization (Scheme 3).

Exposure of the alkenes **15** and **16** to the Grubbs catalyst effects dimerization through olefin metathesis. Olefination of the hydrindanone dimer **23** under Wittig-Horner conditions



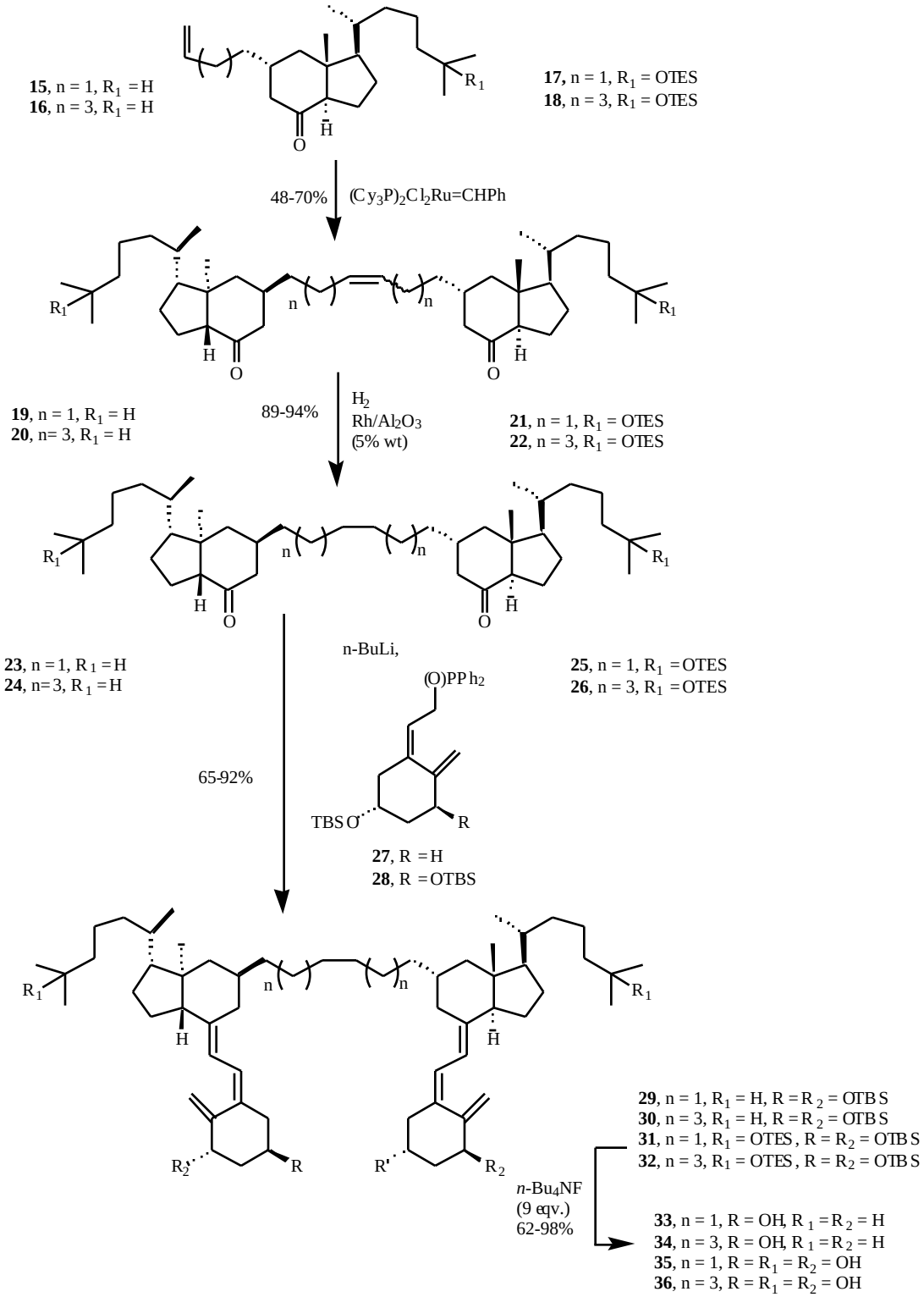

**15**, R = $(CH_2)_2CH=CH_2$
**16**, R = $(CH_2)_4CH=CH_2$
**17**, R = $CH=CH_2$

**Scheme 3**. Synthesis of C-11 functionalized ketones from Grundmann's ketone (**13**).

leads to installation of the vitamin D triene system (Scheme 4).

Based on this short synthesis, the authors propose that construction of dimers is facile and therefore, studies to optimize binding affinity to the VDR will be greatly facilitated.

The synthesis of multivalent ligands can be quite involved; therefore the task of synthesizing molecules to test for increased biological activity is potentially daunting. Boger and colleagues have described a solution phase methodology to generate chemical libraries that can be screened for compounds possessing specific protein-protein interactions [8-10]. It is this methodology that has been successful in identifying a novel homobifunctional molecule




**Scheme 4**. Synthesis of vitamin $D_3$ dimers.

**37** that inhibits angiogenesis and tumor growth *in vivo* by disrupting the binding of matrix metalloproteinase 2 to integrin $\alpha_v\beta_3$ without affecting the individual interactions of each molecule with its respective target [11, 12].

Angiogenesis, a multistep process involving vascular cell activation, matrix degradation, and cell migration, proliferation, and differentiation, when it goes awry, is characteristic of several pathological conditions including arthritis, ocular retinopathy, and tumor growth and metathesis. During tumor-induced angiogenesis, the matrixmetalloproteinase, MMP2, secreted by vascular endothelial cells, plays a crucial role in degrading the extracellular matrix. Integrin $\alpha_v\beta_3$, a heterodimeric cell-surface protein that mediates cellular interactions with the extracellular matrix, is equally important in angiogenesis. Based on a distinct interaction between MMP2 and integrin $\alpha_v\beta_3$ crucial to the angiogenic cascade, a search using combinatorial chemistry was undertaken to identify a molecule that could selectively inhibit the interaction of MMP2 with integrin $\alpha_v\beta_3$ sidestepping the array of side effects associated with non-selective inhibition of other integrin subtypes.

In a process of elimination, the original compound **38** was successively refined to produce **40**, a bivalent derivatized dilysine tetraamide (Fig. (**8**)).

The mechanism of action of **40** is unknown, but Boger *et al*. propose that it may be both direct and indirect. That is, **40** may block the ability of MMP2 to bind to integrin $\alpha_v\beta_3$; however, it is also possible that a secondary site of $\alpha_v\beta_3$,

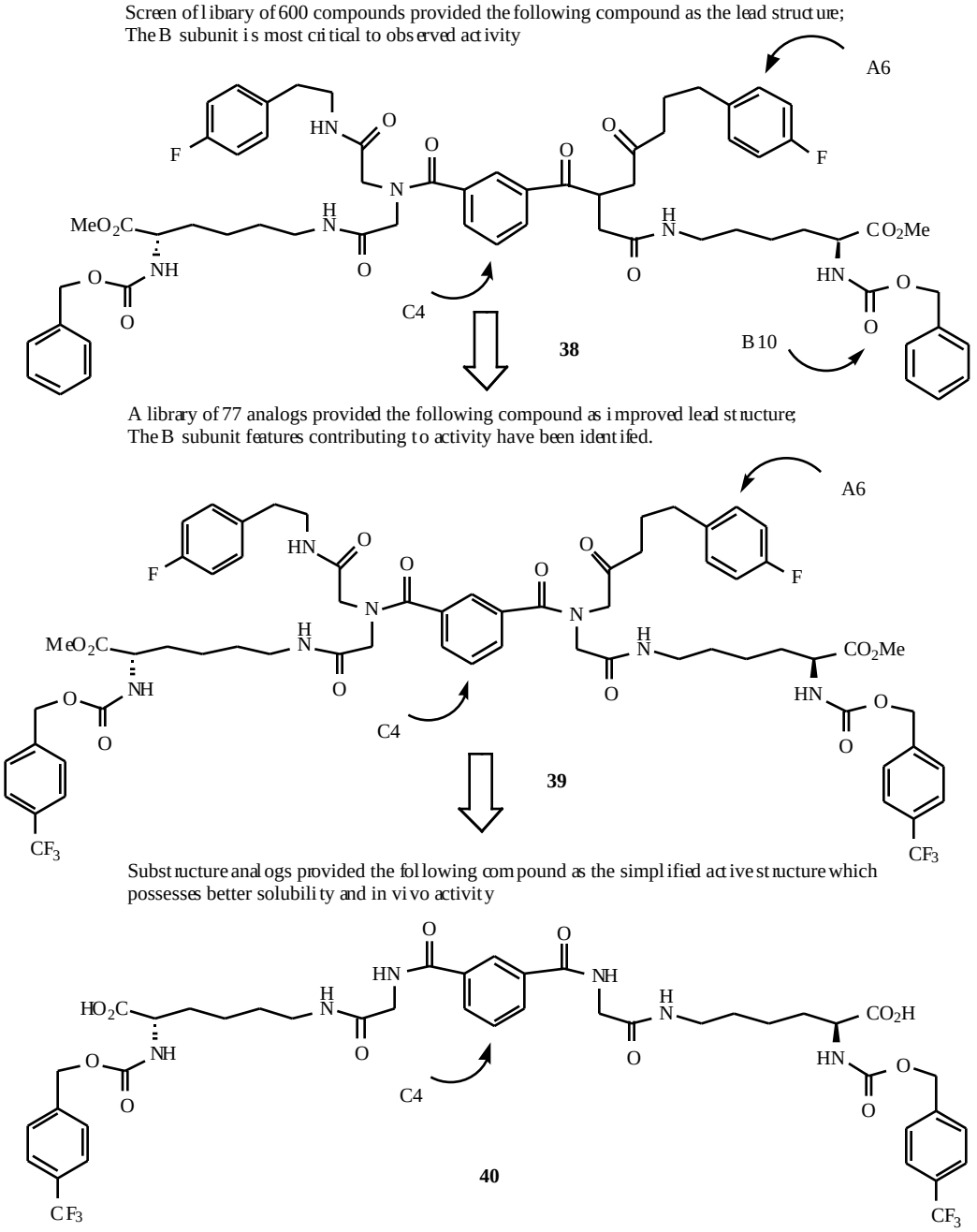



**Fig. (8).** Identification of novel homobifunctional molecules *via* solution phase methodology.

specific for MMP2, when blocked by the mimetic **40**, would prevent $\alpha_v\beta_3$ from recognizing provisional matrices able to support cell survival (Fig. (**9**)).

As an approach to the design of potentially isozyme selective agents for PKC regulation, Wender and colleagues investigated the synthesis of phorbol ester dimers that incorporated two appropriately linked binding subunits possessing high affinity for PKC [13]. The protein kinase C isozymes (PKCs), serine threonine kinases involved in cellular recognition, have been implicated in a wide range of biological processes from learning to tumor promotion and

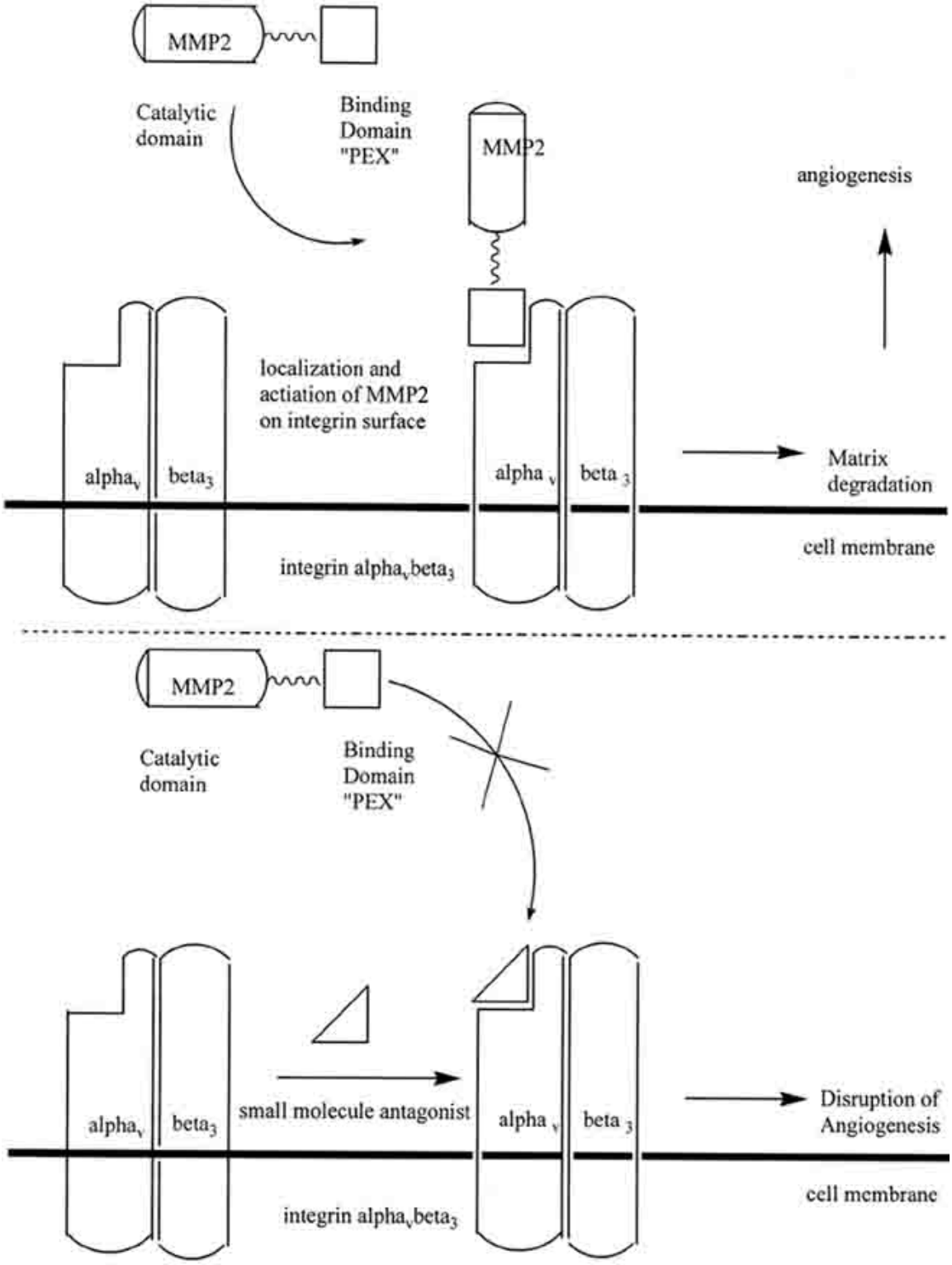



**Fig. (9).** Top: depiction of the MMP2-$\alpha_v\beta_3$ interaction and its role in angiogenesis. Bottom: A small-molecule antagonist can disrupt angiogenesis by inhibiting the localization of MMP2 to the cell surface through binding to $\alpha_v\beta_3$.

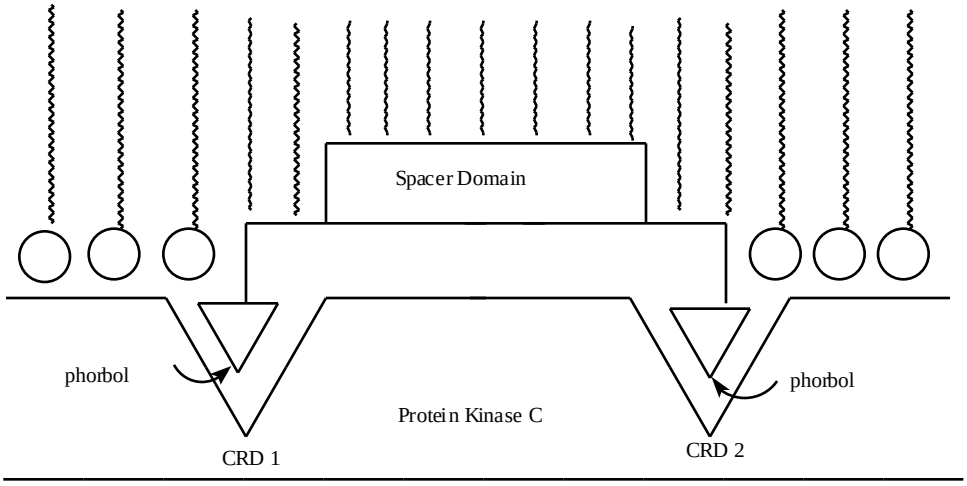
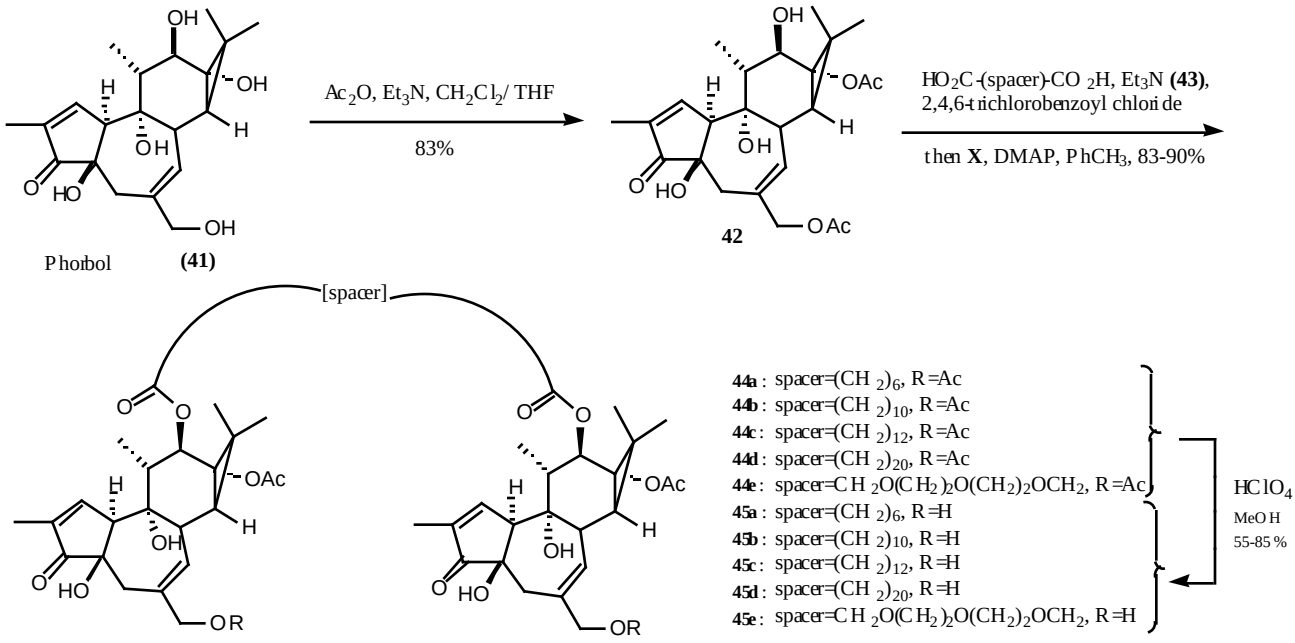



**Fig. (10).** Design concept for isozyme selective compounds.

have been the focus of research directed toward the treatment of cancer, diabetes, and neuropathic pain.

Of the eleven known PKC isozymes, the conventional isozymes (α, βI, βII, γ) are activated by diacyl glycerol (DAG) and calcium, the novel isozymes (δ, ε, θ) by DAG but not calcium, and the atypical (ζ, λ) by neither DAG nor calcium, this class being independent of both activators. Both the conventional and novel PKCs bind DAG and other activators using their first conserved region (C1) that contains two homologous cysteine rich domains, CRD1 and CRD2, also known as C1A and C1B. Prior studies illustrated that a 51-mer corresponding to PKC-γ $_{101\text{-}151}$ (i.e. PKC-γ C1B) bound phorbol esters with affinities and phospholipid requirements similar to the natural protien. Further studies of both CRDs of all conventional and novel PKCs revealed that of the isozymes investigated, only one, PKC-γ, bound phorbol esters at both CRD1 and CRD2 with comparably high affinities. Based on this finding, Wender *et al.* propose a design model that could lead to the development of isozyme selective agents with two binding ligands (one for CRD1 and the other for CRD2 or a secondary site) covalently linked through a tether that correctly positions the two subunits (Fig. (**10**)). The authors suggest that differences in the CRDs or secondary sites among the isozymes would allow for isozyme selectivity.

Based on prior knowledge of the structural features of phorbol esters, indicating that the oxygens at C4 (or C3), C9 and C20 and a lipophiclic domain at C12 are required for recognition at the PKC regulatory domain, two phorbol moieties were linked by esterification at the C12 position to introduce the necessary hydrophobic region without interfering with the atoms directly involved in the binding




**Scheme 5**. Synthesis of phorbol ester dimers Xa-e using a modular synthetic approach.

interaction. A series of phorbol dimers with linkers of varying lengths were synthesized as outlined in Scheme 5. Upon exposure of phorbol (**41**) to acetic anhydride and triethylamine, the 13,20-bisacetate **42** was achieved. Coupling of **42** with the bis-mixed anhydride, obtained by subjecting the diacid **43** to the Yamaguchi method, followed by a final deprotection yielded dimers **45a-e**.

Based on initial results of a conventional binding assay investigating inhibition of [$^3$H]-phorbol 12,13 dibutyrate binding to a PKC isozyme mixture, dimers **45a-d,** possessing the simplest polymethylene linkers, exhibited the most potent binding, in the low nanomolar region. Dimer **45e**, on the other hand, incorporating a glycol based tether, showed relatively poor binding. Upon further investigation of compounds **45a-d**, $K_i$ values in the picomolar region were obtained. Competition binding assays resulted in values of 4.5 nM, 0.1 nM. 0.4 nM and 2.7 nM for dimers **45a, 45b, 45c**, and **45d** respectively, indicating an optimum linker length of 10 to 12 methylene groups. A standard assay for enzymatic activity illustrated that the dimers promoted phosphorylation of a myelin basic protein peptide fragment (MBP), suggesting that binding to PKC was accompanied by a normal catalytic response. These results support that the dimers activate PKC. Wender *et al.* propose that the differential binding of the two ligands of the dimer to specific sites of PKC provides the basis for an approach to the design of PKC isozyme selcetive binders.

Schreiber and Crabtree, who originated the concept of "chemical inducers of dimerization", have focused on the use of CIDs to enhance the activity of a protein by bringing it into close proximity with another protein [14, 15]. Since an interaction must be maintained on both ends of the dimer intermediate, the authors claim that specificity is enhanced.

One example which demonstrated the activity of CIDs is FKCsA, a heterodimeric CID comprised of a protein-binding surface from FK506 and another from cyclosporin A (CsA), so that it is able to bring into close proximity FKBP12 (the FK506 binding protein) and the Fas receptor and thus control subcellular localization and signaling (Fig. (**11**)) [16].

FKCsA was synthesized as outlined in Scheme 6. Cyclosporin was modified by introduction of an allylic bromide to give **46** which was used to alkylate a malonate derivative ultimately introducing a carboxylic acid residue for linkage to FK506. The coupling partner was synthesized from **48**, the succinimidyl carbonate derivative of FK506. It was then coupled with hexamethylene diamine to give **49**. Coupling of fragments **47** and **49** under dehydrating conditions gave the CID **50**.

Experimental data illustrated that the FKCsA was able to bind to FKBP12 and the chimeric Fas receptor, bringing the two proteins into contact, thus activating the associated signaling pathway. Up to three Fas domains were recruited to a single membrane-docking protein by FKCsA. The FKCsA was able to conditionally equip a cytoslic protein with a membrane localization domain and therefore induce its membrane localization (Fig. (**11**)).

In another example, Screiber and Crabtree utilize these CIDs as a transcriptional on-off switch [17]. Using FK1012, a dimeric form of FK506, Schreiber and Crabtree were able

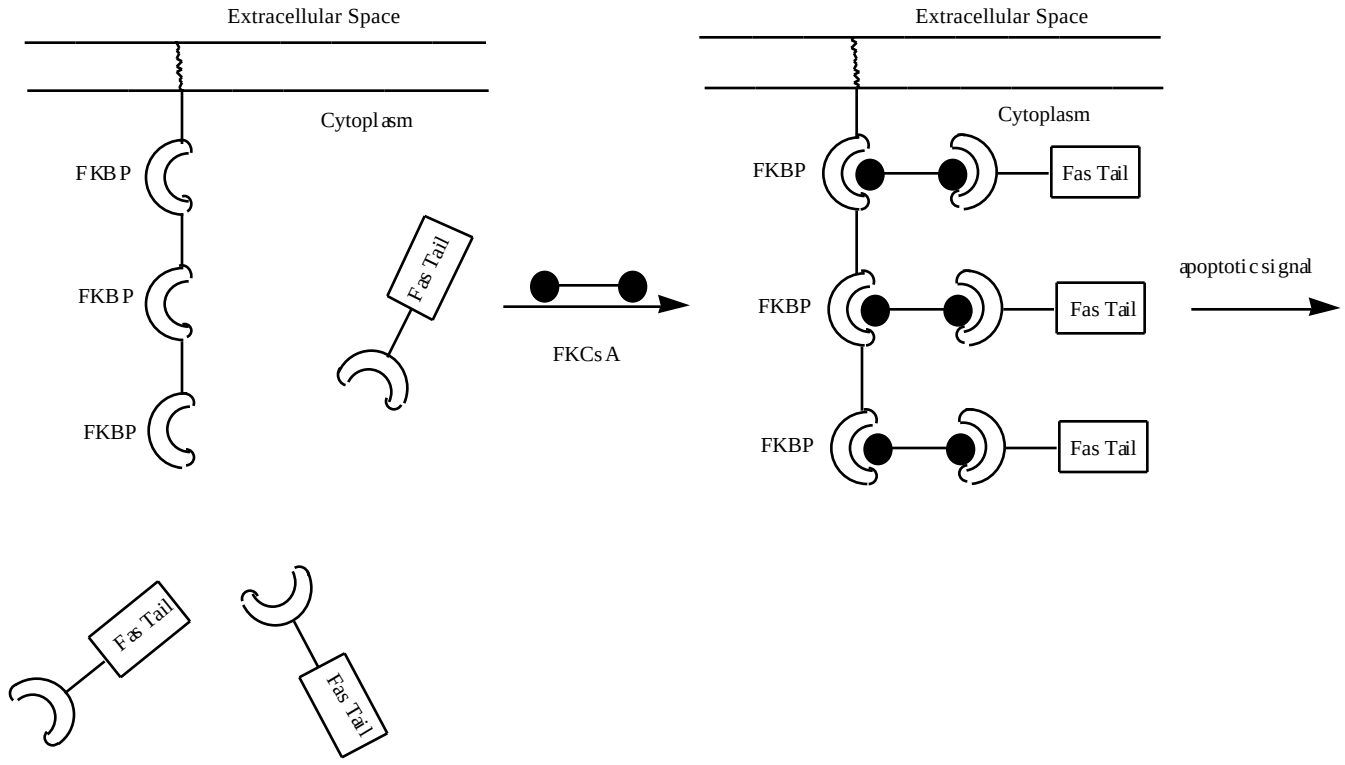
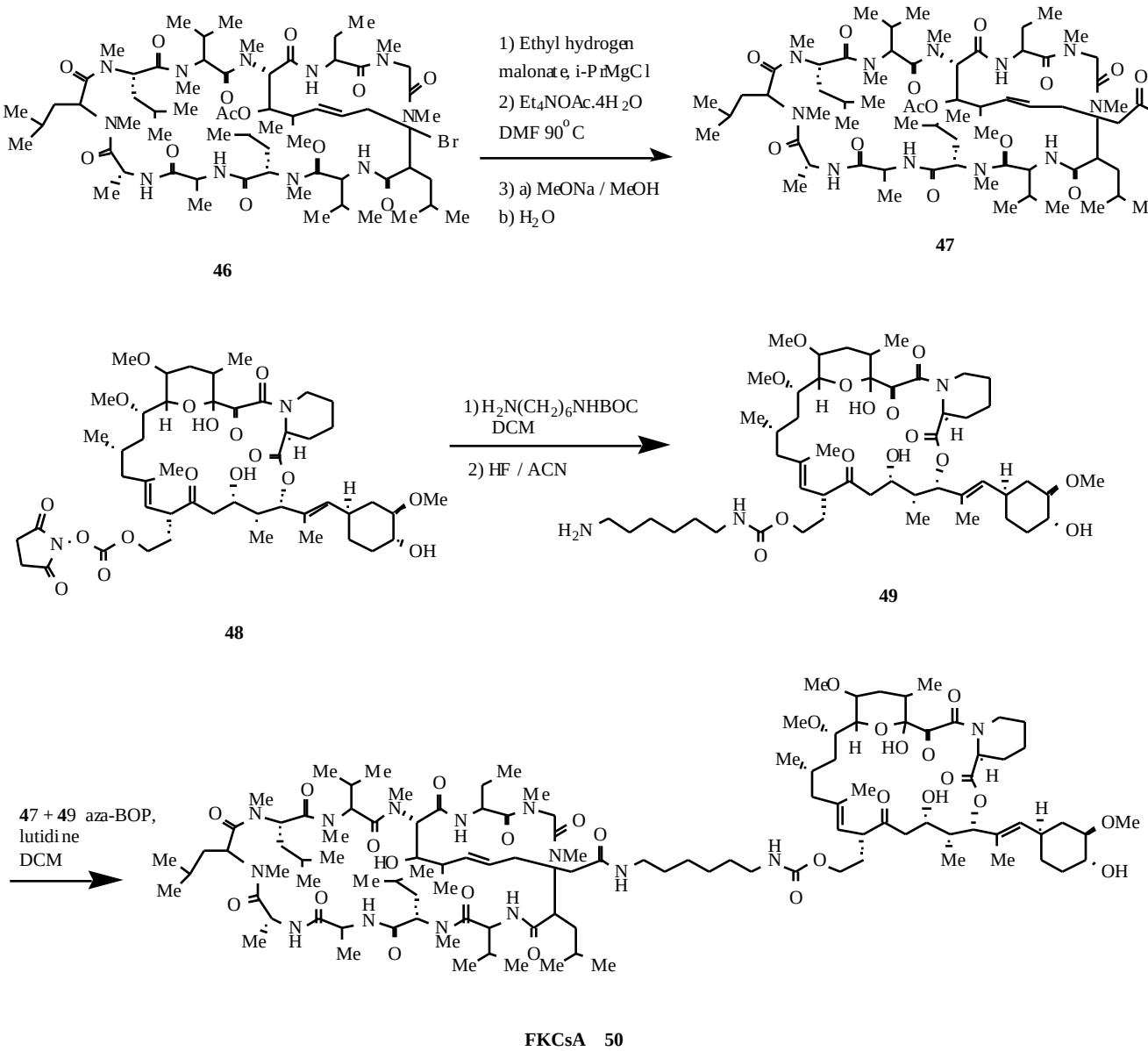



**Fig.** (**11**). Illustration of FKCsA-inducible membrane translocation.




**Scheme 6**. Synthesis of FKCsA.

to direct interactions between proteins linked to the ligand receptor FKBP12 (Fig. (**12**)).

The authors report that FK1012 regulates transcription by inducing recruitment of a transcriptional activation domain to a promoter containing FKBP12. In addition, the monomeric FK506, which is unable to bring the two domains into proximity, competes with FK1012 and eliminates the interaction between the DNA binding and activation domains. The authors report a six to ten fold increase in the rate of transcription upon addition of FK1012, followed by a 64% reduction in the induced rate upon addition of FK506. Experimental data also indicated that in order to maintain ongoing transcription, the transcriptional activation domain must remain in contact with the DNA binding domain. The FK1012 dimer facilitates continued interaction between the two domains.

This focus on the use of FK1012 dimers led to the investigation of their ability to control insulin and PDGF receptor signaling [18]. Schreiber and colleagues were successful in using FK1012 to conditionally induce dimerization or oligomerization in proteins containing FKBP fused to either the insulin or the PDGFβ receptor. Chimeric, myristoylated receptor tyrosine kinases that attach to the plasma membrane intracellularly and lack the extracellular ligand domains were engineered to test whether FK1012 would trigger dimerization or oligomerization, thus activating the protein. Not only was the chimeric PDGFβ receptor activated by FK1012, albeit more slowly than the wild type receptor, but also its ability to signal through its down stream signaling pathways was not lost.

Among other physiological and pathological processes, PDGF receptor signaling is involved in neoplastic transformations and embryogenesis. PDGFβ receptor

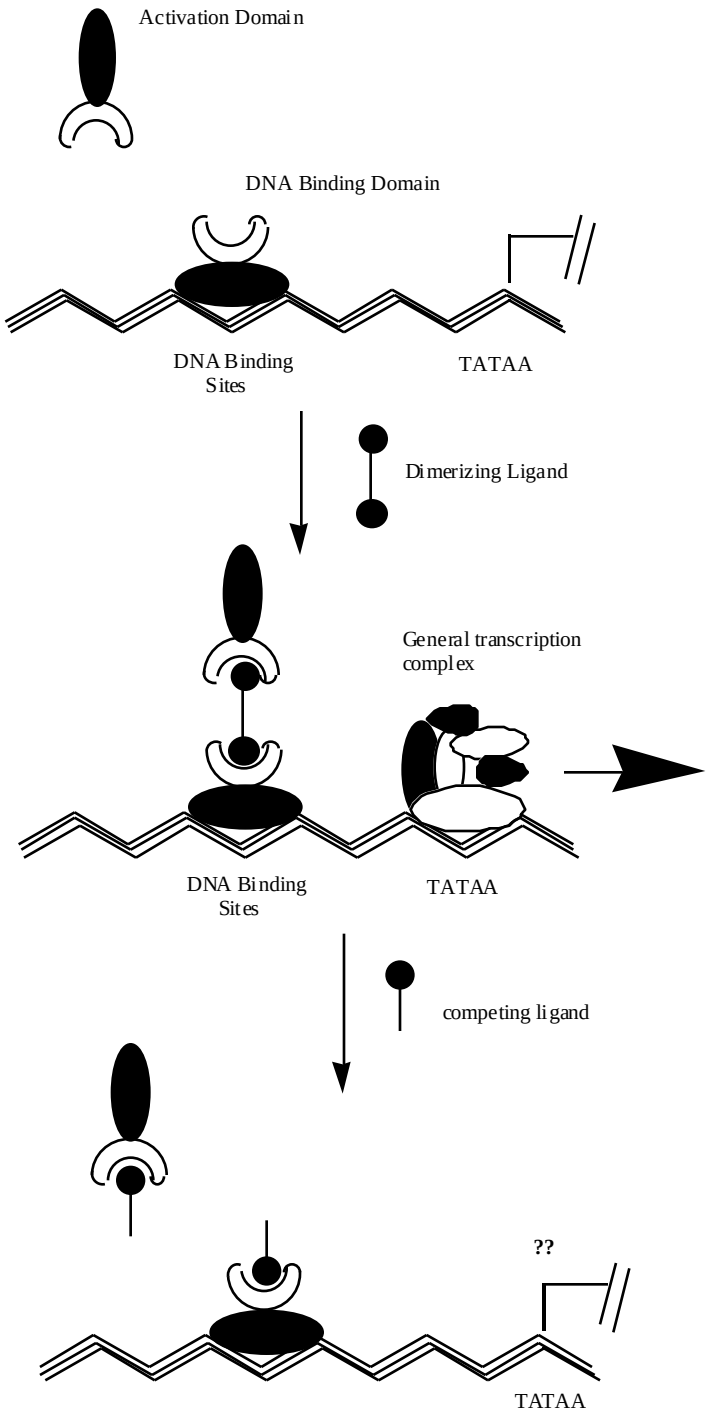
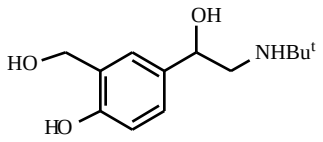



**Fig. (12)**. Illustration of recruitment of an activation domain to a DNA-binding domain with a dimeric ligand, and dissociation of the activation domain with a monomeric ligand.

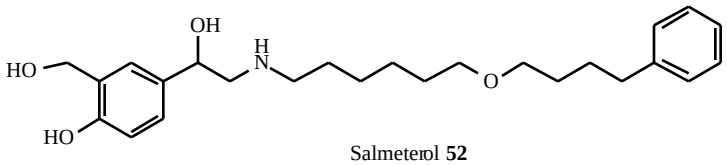
signaling is essential for development of the cardiovascular, hematopoietic and renal systems in mice. Studies showed that, in the presence of the engineered PDGFβ receptor, FK1012 (1μM) was able to induce the formation of loose mesenchymal tissue typical of ventral mesoderm.

Screiber *et al*. also report success with FK506 dimers for the activation of the insulin receptor despite the complexity of insulin binding, which is believed to induce a conformational change leading to receptor activation. These results support that dimerization or oligomerization of the β subunits of the insulin receptor are sufficient to activate receptor signaling.

In a later publication, Schreiber and colleagues exploit the use of CIDs to aid in the understanding of the TGF-β signaling pathway [19]. Transforming growth factor β 1, 2, and 3, which are secreted, dimeric polypeptide signaling factors, participate in various activities on different cells such as induction of cell cycle arrest in the G1 phase in epithelial




Salbutamol **51**




Salmeterol **52**

**Fig. (13)**. Structures of the β-adrenoreceptor agonists salbutamol (**51**) and salmeterol (**52**).

cells and upregulation of extracellular matrix proteins such as fibronectin and plasminogen activator inhibitor 1. TGF-β receptors are activated by binding to specific transmembrane serine/threonine kinase receptors. Schreiber *et al.* were able to study interactions between cytoplasmic tails of two of these kinase receptors, TGF-βRI and TGF-βRII using CIDs. Upon interaction of these two proteins, Smad nuclear translocation is induced. The authors have also shown that homo-oligomerization of the TGF-βRI cytoplasmic tails by CIDs induces activation of TGF-β signaling.

This idea of increased affinity based on multiple binding sites has been put to practical use in the form of salmeterol (**51**) (Fig. (**13**)). Salmeterol, a β-adrenergic receptor (β-AR) agonist is used in the treatment of bronchial asthma, a complex disease characterized by episodic bronchoconstriction, airway hyperreactivity, inflammation and muco-ciliary abnormalities.

Before salmeterol, one of the major obstacles in the treatment of this disease was the absence of a long-acting compound. Available drugs had a duration of activity of no more than four to six hours, preventing prolonged, overnight relief. To overcome this problem, research was directed at synthesizing a molecule having two major sites of interaction, one at the active site and another nearby [20]. The hope was that this modified architecture would not only effect the necessary pharmacological activity, but at the same time allow the molecule to anchor itself to the receptor, facilitating repeated stimulation of the active site of the receptor, prolonging duration of activity. Based on the structures of previous compounds such as salbutamol (**51**) (Fig. (**13**)) studies were undertaken to design a molecule with a hydrophobic chain that would serve as an additional anchor to its cellular target. SAR of N-aralkyloxyalkyl analogues of salbutamol revealed that the duration of action of the desired compound is dependent upon the overall lipophilicity of the molecule, the length of the side chain, and the presence and position of the oxygen atom. Salmeterol emerged as the compound showing greatest increase in duration of activity. The molecule, 25 Å in length as compared to salbutamol, which is only 11Å long, is 10,000 times more lipophilic than salbutamol and can therefore rapidly partition into the outer phospholipid monolayer by a factor of approximately 30,000:1. Its increased lipophilicity, however, is not solely responsible for its drastically increased duration of activity. Based on experimental studies, Johnson *et al.* have shown that salmeterol persists at the receptor even after washout because it is anchored at a site separate from, but near the active site (Fig. (**14**)).

According to Johnson and colleagues, this anchoring is not due simply to non-specific lipophilic interaction of the drug within the membrane, but rather specific interaction with a distinct "exosite" domain of the $\beta_2$AR [21]. This was confirmed by studies showing that changes in the position of the oxygen atom of the side chain of salmeterol resulted in a decrease in duration to less than thirty minutes versus the twelve plus hours observed with salmeterol, despite the fact that the change in oxygen position has no effect on lipophilicity.

Site directed mutagenesis was used to determine that the "exosite" is a discrete sequence within the $\beta_2$-AR, indicating that the prolonged activation results from a structurally

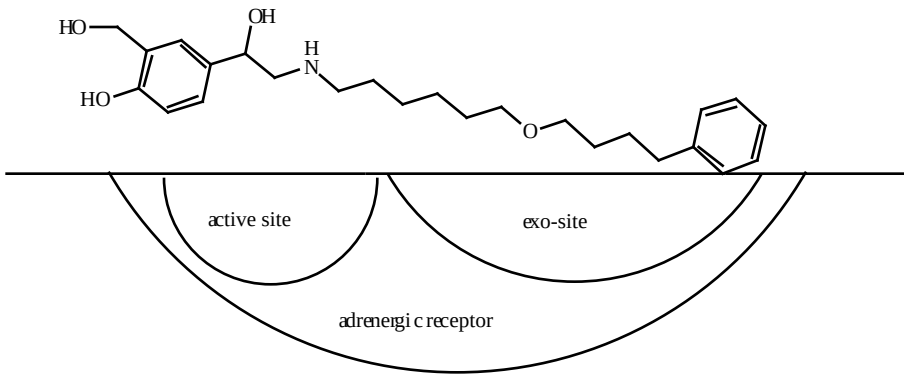



**Fig. (14)**. Proposed mechanism of action of salmeterol.

related interaction with the $\beta_2$-AR [21]. Elimination of the "exosite" results in loss of duration of activity of salmeterol, but not loss of ability to bind to the active site. Johnson *et al*. propose that salmeterol binds not only to the receptor active site, but also to this "exosite" in the $\beta_2$-AR. This secondary binding allows the drug, in the presence of $\beta_2$-AR antagonists, to remain in close contact with the receptor and to persist at the site, thus increasing the probability of receptor activation and duration of activity.

With this and many other examples in nature where multiple binding sites lead to increased affinity, the number of attempts to exploit this strategy in the study of a variety of biological processes has increased considerably. Crabtree and Wandless have proposed a strategy for the modulation of the binding affinity of small-molecule ligands reminiscent of mechanisms used in nature where small molecule ligands, through binding to an endogenous protein and increasing the surface area for interaction, enhance their affinity for target proteins [22]. This is exemplified by the immunosuppressive FK506-FKBP complex that binds tightly to and inhibits the activity of calcineurin, a ubiquitous protein phosphatase widely used in signaling. Alone, FK506 displays no such inhibitory influence on calcineurin. Based on this model, Crabtree and Wandless have proposed the synthesis of bifunctional molecules that can simultaneously bind two separate proteins by chemically linking to a ligand of interest another small molecule that has a high affinity for a secondary protein. This secondary protein now serves as a presenter molecule, bringing the ligand to the target protein. As the trimeric complex, comprised of the ligand, small molecule and presenter, approaches the target, the nonselective interactions of the presenter molecule and the target may either be favorable, thus increasing the affinity of the ligand for the target, or unfavorable, resulting in reduced affinity.

Crabtree and Wandless demonstrated their technique using as presenter molecules two members of the FK506-binding protein family, FKBP12 and FKBP52. The SH2 domain of the Fyn tyrosine kinase was the designated target as its structure is known and ligands, with some affinity for it, widely available. The ligand featured in this particular study was the tertapeptide, phosphotyrosyl-glutamyl-glutamyl-isoleucine. The two bifunctional molecules synthesized incorporated FK506 and a smaller synthetic analog of FK506 (SLF) respectively into the phosphopeptide (pYEEI) (Fig. (**15**)). Linkers were designed on the basis of three-dimensional structures of FKBP12, FKBP52 and the Fyn SH2 domain surface. Results of a binding assay showed

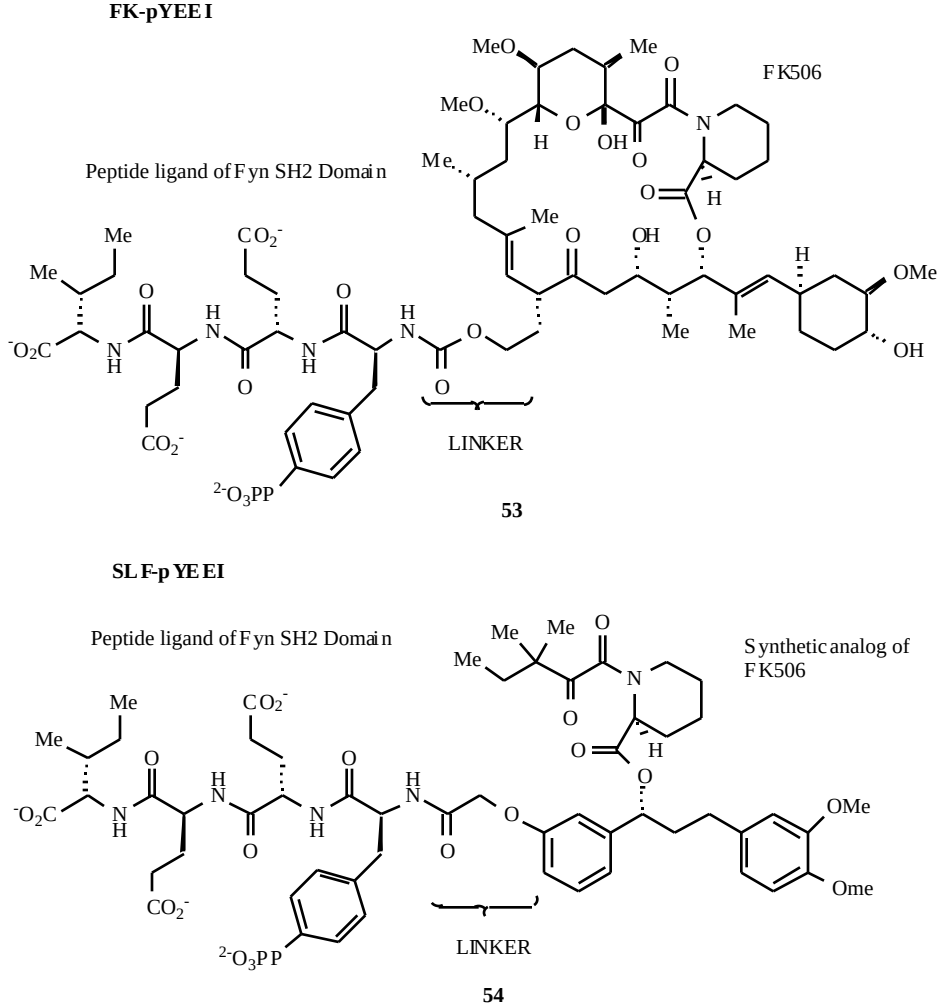



**Fig. (15)**. Structures of FKpYEEI and SLFpYEEI. The C21 allyl group of FK506, which is required for calcineurin binding, was used to link the pYEEI peptide, effectively eliminating any immunosuppressive activity of the FKpYEEI molecule.

that both bifunctional molecules, FKpYEEI (**53**) and SLFpYEEI (**54**), support formation of a trimeric complex with the SH2 domain and either FKBP12 or FKBP52, though FKpYEEI formed the complex more efficiently with FKBP52.

Crabtree and Wandless also report that the FKpYEEI when bound to the FKBP52, interacts more effectively with the SH2 domain than in the absence of the presenter molecule as a result of the binding of FKpYEEI to the FK506 binding site pocket of FKBP52. The FKpYEEI bound to FKBP52 with $K_D$= 150nM. SLFpYEEI bound more strongly to FKBP12 than to FKBP52 ($K_D$= 60nM vs. $K_D$=5000nM respectively). In the presence of FKBP52, the affinity of FKpYEEI for the SH2 domain is enhanced by a factor of three, the $IC_{50}$ having a value of 750 nM ($K_D$=340nM) in the absence of FKBP52 and a value of 250 nM ($K_D$=120 nM) when the presenter molecule is bound to it. The authors suggest that the increase in affinity is a result of the increased surface interactions between the FKBP52 and the SH2 domain that enhance the stability of the complex.

On the flip side, the second bifunctional molecule synthesized by Crabtree and Wandless, SLFpYEEI, when bound to FKBP12, displayed a decreased affinity for the Fyn SH2 domain. Based on results of a binding assay in which increased concentrations of FKBP12 led to a decrease in binding to the SH2 domain, FKBP12 decreases the affinity of the bifunctional molecule for the target. In the presence of bound FKBP12, the $IC_{50}$ of SLFpYEEI for the Fyn SH2 domain increased 6 fold from 0.25 to 1.5 μM and a 5.5 fold increase was observed in the $K_D$ from 180 nM to 1.0 μM. The authors suggest that unfavorable interactions derived from steric hindrance or electronic repulsion between the surface of the FKBP12 and the Fyn SH2 domain destabilize the complex, resulting in a decrease in affinity.

A later study by Wandless *et al.* focused on the advantages of bifunctional ligands whose affinity for a specific ligand could be decreased by a presenter molecule [23]. Wandless *et al.* suggest that these ligands, whose activity levels are dependent upon cellular context, could be key in achieving specificity of action. According to Wandless and colleagues, such ligands could prove useful in the detoxification of naturally cytotoxic bifunctional molecules where the presenter protein could direct selective antibiotic activity. In this study, isotope-edited NMR spectroscopy was used to investigate possible explanations for the reduction in binding affinity for the SH2 domain observed in the case of the SLFpYEEI-FKBP12 complex. Comparisons were made between the $^{1}H/^{15}N$ HSQC spectra of the SLFpYEEI-FKBP12 complex and the FKBP12-SLF complex. The most significant differences occurred at a site adjacent to the binding site of FKBP12, suggesting an interaction between the pYEEI peptide and FKBP12. Wandless *et al.* postulate that an attractive interaction between the pYEEI and the FKBP12 may result in reduced affinity. Another explanation may be that FKBP12 induces conformational changes in the pYEEI peptide, preventing it from binding to the SH2 domain, though no evidence is offered to support this idea. The construction of heterodimeric ligands to increase binding affinity has not been limited to this single area, however. In fact, the principle has been applied to the study of various biological

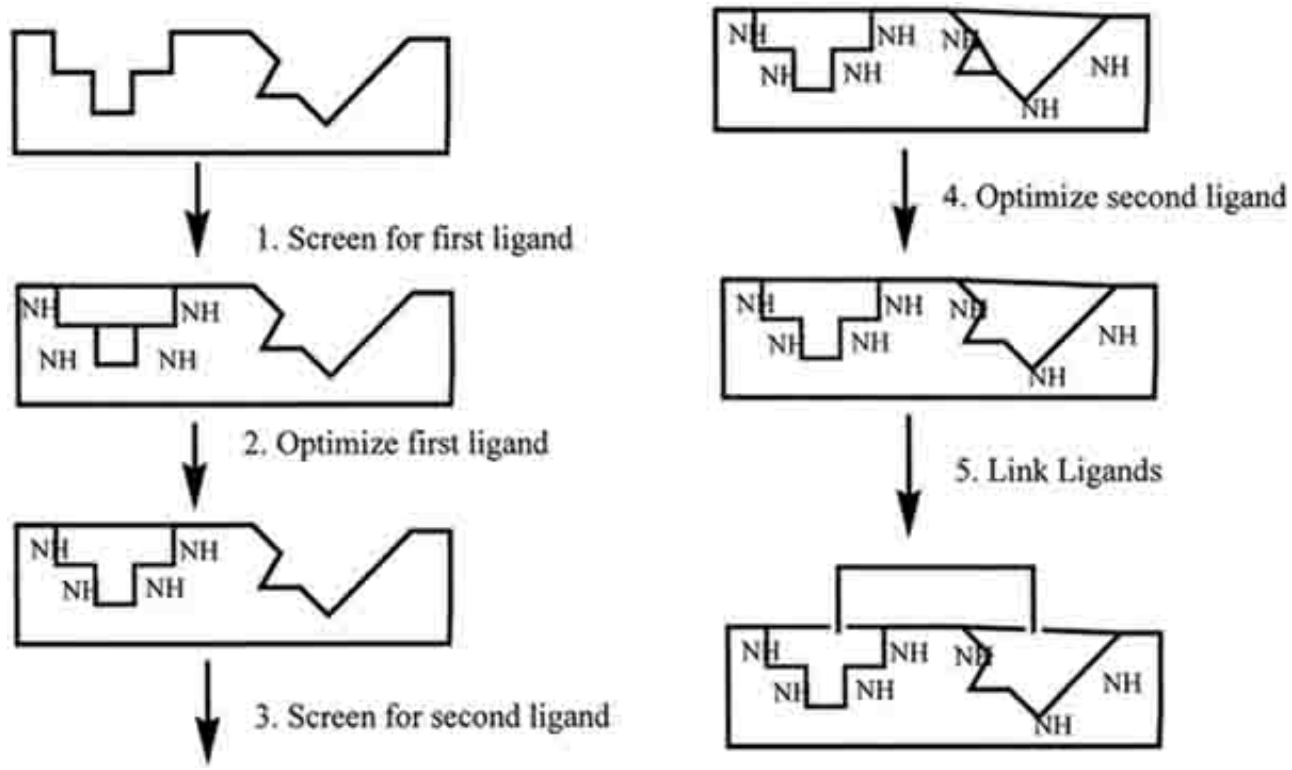
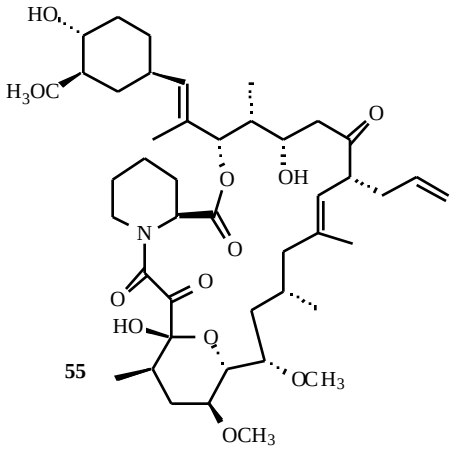



**Fig. (16)**. An outline of the SAR by NMR method.

systems and has led to the development of several techniques to aid in the simplification of the design of these bifunctional molecules. One method exploited by Fesik and co-workers for identifying high affinity ligands to aid in the drug discovery process is "SAR by NMR", structure-activity relationships by nuclear magnetic resonance [24]. As eluded to in its name, this method involves the screening of a library of small molecules in search of those which bind, even weakly, to proximal sites on a protein, by monitoring perturbations of the amide chemical shifts of the $^{15}$N-labeled protein upon the addition of potential ligands. Once two compounds that bind to the same protein have been identified, the next step is to link the two, producing a compound with a much larger binding energy than each individual. Experimentally derived structural information is used to guide the design of the linkers between molecules.

In the first step of the process, a search is undertaken for molecules that bind to the protein of interest (Fig. (**16**)). If binding occurs upon addition of a ligand to an $^{15}$N-labeled protein, a change in the $^{15}$N- or $^{1}$H-amide chemical shift in two dimensional $^{15}$N-heteronuclear single-quantum correlation ($^{15}$N-HSQC) spectra is observed. Based on the ease with which such spectra are obtained, large libraries of compounds can be efficiently screened to identify molecules with potential binding capabilities. Once a lead molecule is identified, analogs are screened to optimize binding to this site. Then, using either the original screen or a screen conducted in the presence of the first optimized fragment, a second ligand, which binds to a nearby site, is sought. In this second screen, a different set of amide chemical shifts is monitored for changes. Based on this second set of shifts, the approximate location of the second ligand can be identified. Again, analogs of this second ligand are tested in order to optimize binding. Upon identification of two optimal fragments, NMR spectroscopy or x-ray crystallography is employed to elucidate their location and orientation in the ternary complex. Based on this structural information, linkers are designed to bridge the two compounds resulting in the synthesis of a high affinity ligand with an extended binding surface.

The authors have demonstrated the usefulness of this technique with a range of proteins. They first illustrated the use of SAR by NMR in the discovery of ligands that bind to the FK506 binding protein (FKBP), a protein, which when bound to the potent immunosuppressant FK506 (55) (Fig. (**17**)), inhibits calcineurin (a serine-threonine phosphatase) thereby blocking T-cell activation [24].

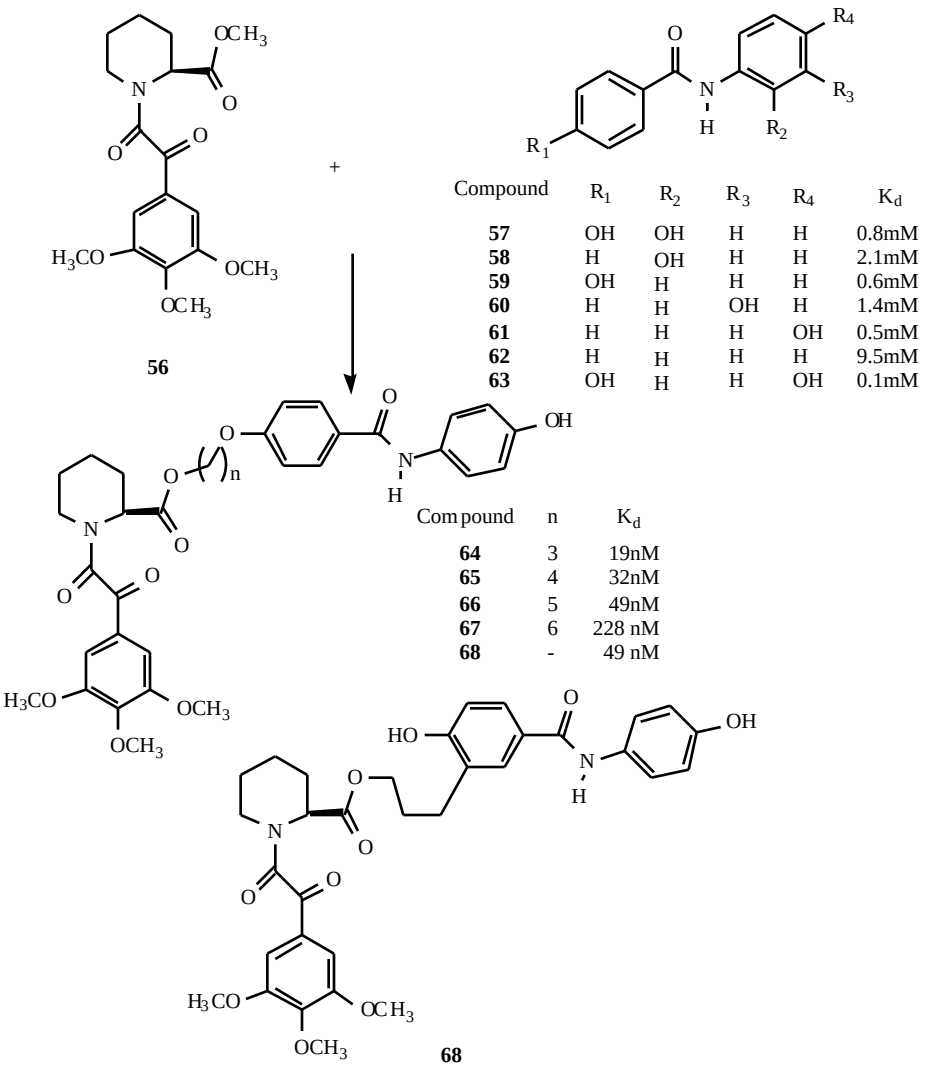



**55**

**Fig. (17)**. Structure of the potent immunosuppressant FK506.

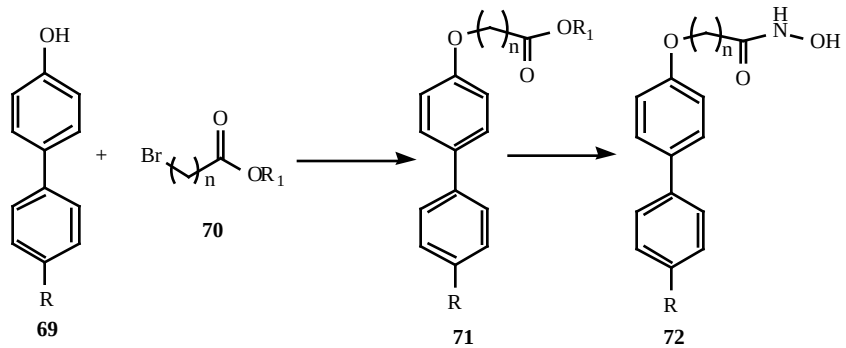
By screening FKBP against a library of potential ligands, a variety of compounds were observed to exhibit weak binding to FKBP (Scheme 7). A trimethoxyphenyl pipecolinic acid derivative **56**, the compound demonstrating the highest binding affinity to the protein, was shown to bind to FKBP via the same site as the pipecolinic acid moiety of FK506 based on amide chemical shift changes of FKBP upon binding. Following the SAR by NMR strategy, a second series of screenings were carried out in the presence of saturating amounts of **56** to identify molecules that interact with FKBP at a nearby site. Based on changes in $^{15}$N-HSQC spectrum of uniformly $^{15}$N-labeled FKBP bound to **57**, the benzanilide derivative **57** was determined to bind with an affinity of 0.8mM to a site on FKBP near the binding site for **56**. An attempt was made to optimize this second compound, and based on SAR of a series of analogs of **57**, a ligand **63** ($R_1$, $R_4$ = OH, $R_2$, $R_3$ = H) with an affinity of 100μM for the second site of FKBP was synthesized. The final step lay in designing a linker to connect the two ligands. Using isotope-filtered NMR studies to construct a representative ternary complex of the individual ligands and FKBP, linkers were designed that would not only perform the necessary task of connecting the two compounds, but would do so without interfering sterically with the protein. The binding affinities of the linked compounds **64** to **68** were measured in a fluorescence based assay and the authors report a drastic increase to nanomolar levels of affinity for FKBP. The length of the linker was varied to optimize the binding affinity of the dimer. The heterodimer **67**, linked by a tether of six carbons, had a $K_D$ value of 228nM compared to the ligand linked by only three carbons ($K_D$= 19 nM). The nuclear Overhauser effects (NOEs) observed in the NMR structure of the FKBP-heterodimer complex were compared to those observed in the ternary complex composed of **56**, **63** and FKBP, and it was ascertained that the linked compounds bound to the same sites on FKBP as the untethered compounds.

Fesik *et al*. have demonstrated the versatility of the SAR by NMR method by applying it to the discovery of yet another set of ligands, this time directed towards the inhibition of stromelysin, a matrix metalloproteinase involved in matrix degradation and tissue remodeling [25]. A member of a family of zinc-dependent endoproteinases, stromelysin, when overexpressed or dysregulated, is associated with events such as arthritis and tumor metastasis. Any ligand that could potentially inhibit stromelysin would have to inhibit autolytic degradation, be soluble enough to saturate the protein, and not produce destabilizing steric interactions with other binding sites on the protein. Based on previous knowledge of the structural characteristics of MMP inhibitors, acetohydroxamic acid, which contains the active hydroxamate moiety able to chelate to the zinc in the stromelysin active site, was tested for its ability to bind to stromelysin and inhibit autolytic degradation. Despite its low affinity for the metalloproteinase, it was chosen as the first ligand based on its effective inhibition of stromelysin activity at ligand




**Scheme 7**. Summary of the SAR by NMR method as applied to FKBP.

concentrations of > 100mM, its small size, and its high solubility in aqueous buffers. In the search for the second ligand, a series of hydrophobic compounds were screened in the presence of saturating amounts of acetohydroxamic acid. The choice of compounds to screen stemmed from structural studies of stromelysin that reveal its large, hydrophobic $S_1'$ binding site. Derivatives of a series of weakly binding biphenyls and biphenyl analogs were synthesized in an attempt to optimize binding to stromelysin. Fesik and co-workers report the highest affinity biphenyl analogs to contain a *para n*-propyl or a *meta* $CH_2CN$ substituent. The two fragments of the heterodimeric complex having been identified, focus was directed at determining the ternary structure. Based on NOE experiments for two sets of ligands, it was verified that the ligands bind to the $S_1'$ pocket of stromelysin. Linked compounds were then synthesized following SAR (Scheme 8).




*R groups varied from H to CN; $R_1$ groups varied from methyl to ethyl to t-butyl; n varied from 1 to 3 to 4

**Scheme 8**. Synthesis of linked compounds.

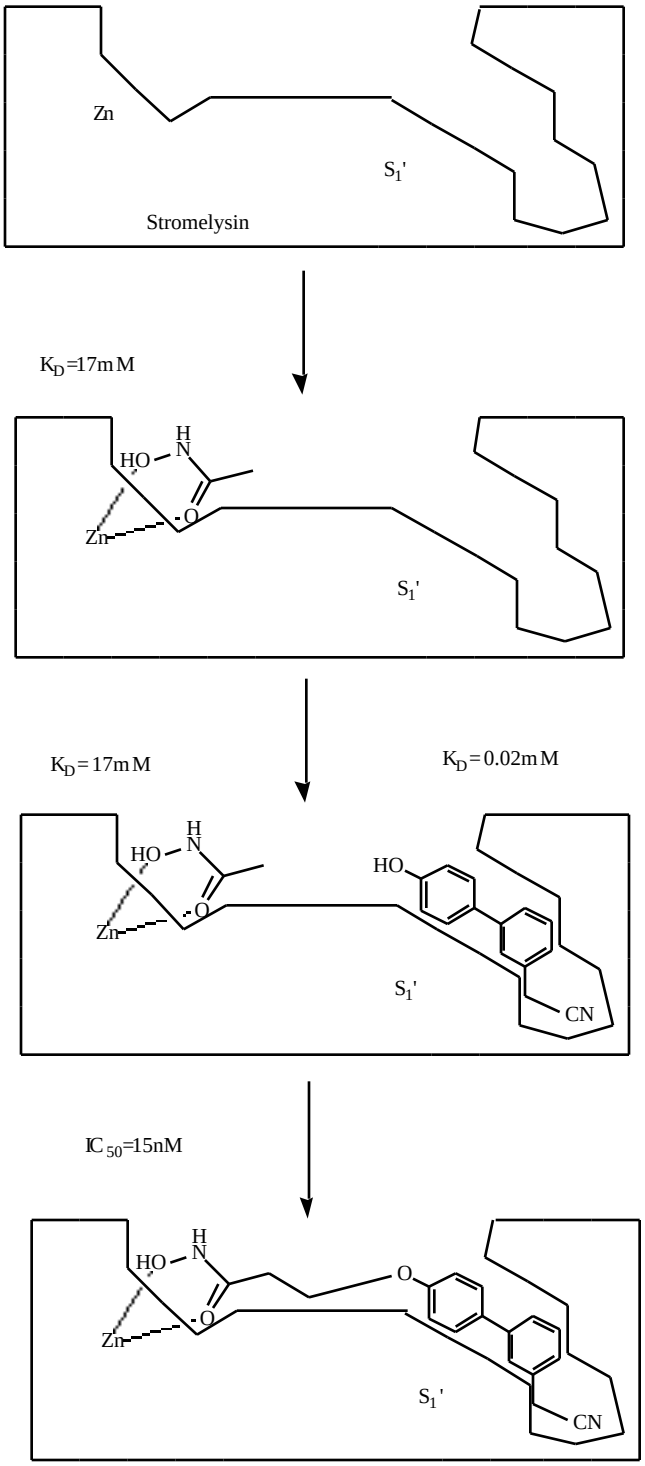



**Fig. (18)**. A Summary of the SAR by NMR method as applied to the discovery of stromelysin inhibitors.

Results of a stromelysin inhibition assay indicated that the linked compounds showed enhanced activity over the untethered ones. An enhancement of activity of up to 1000 was observed. NMR data was again relied upon to verify that the heterodimer bound to the same site in stromelysin as the individual fragments. By their SAR by NMR method (Fig. (**18**)), Fesik *et al*. have been able to synthesize inhibitors of stromelysin that are significantly more potent than previously designed peptide inhibitors containing an isobutyl group that binds to the same $S_1'$ site. They attribute the greatly increased affinities of the biaryl compounds to the fact that the biphenyl moiety of these compounds sits deep in the $S_1'$ pocket as opposed to the leucine side chain of the leucine analogs which sits at the "top" of the pocket.

Yet another example of the versatility of this SAR by NMR method is the discovery of inhibitors that block DNA

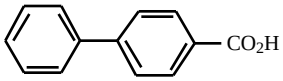
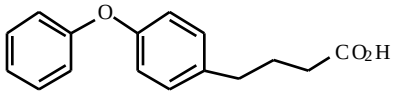
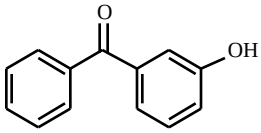
| No. | Compound | $K_D$ (mM) | Binding Site | Inhibition of DNA Binding |
|---|---|---|---|---|
| **73** |  | 2.5 | DNA recognition helix | + |
| **74** |  | 1.9 | DNA recognition helix | + |
| **75** |  | 0.6 | beta-barrel | - |

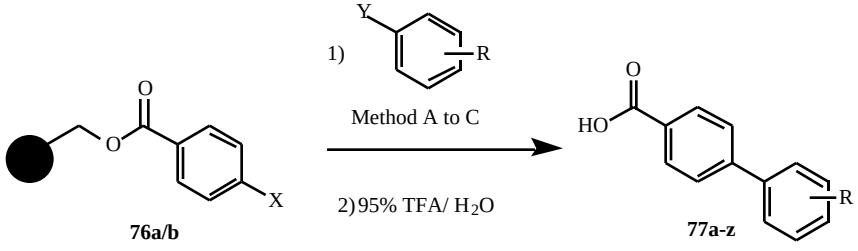
**Fig. (19)**. Initial ligands for the E2 protein discovered using SAR by NMR.

binding of the human papillomavirus E2 protein [26]. A group of small DNA tumor viruses known as papilloma viruses cause benign, proliferative lesions of the skin or mucosa, and certain strains have been implicated in the development of cervical carcinomas and anogenital warts. Regulation of transcription of several viral genes is controlled by the DNA-binding E2 protein. E2, in conjunction with the E1 protein, is therefore required for viral replication. Using uniformly $^{15}N$-labeled E2 DNA-binding domain, a series of small molecules were screened. Three classes of compounds were observed to induce changes in the amide chemical shifts of two distinct binding sites in the E2 protein (Fig. (**19**)). Biphenyl carboxylic acids **73** and biphenyl ether carboxylic acids **74**, upon binding, caused changes in one set of amide shifts, while those compounds 75 with a benzophenone moiety, but lacking carboxy functionality, caused changes in another set of amide chemical shifts, indicating that this class of molecules binds to a different site in the E2 DBD than the first two classes. A filter-binding assay was employed to investigate the ability of each class of compounds to inhibit the binding of E2 to DNA. Of the three classes of molecules identified to bind weakly to the E2 DBD ($K_D$'s from 0.6-2.5 mM), the benzophenone derivatives did not inhibit binding of DNA either by directly blocking the DNA binding site or by disrupting the homodimerization of E2.

Two-dimensional isotope-edited NMR experiments were then performed on a complex of E2 and a biphenylcarboxylic acid derivative to confirm the ligand binding site and to provide structural information on the orientation necessary for inhibition of DNA binding. A series of biphenyl carboxylate analogs were then synthesized as outlined in Scheme 9 to investigate the SAR of the compounds.

Fesik *et al.* report a 3′,5′dichloro-substituted derivative which shows a 40-fold enhancement in activity ($K_D$=0.06 mM, $IC_{50}$=0.15 mM). In the case of the biphenyl-ether derivatives, upon screening a series of commercially available analogs, it was observed that the short linkers between the biphenyl moiety and the carboxylic acid group resulted in reduced binding affinity. Consequently, several compounds were synthesized as outlined in Scheme 10 that lengthened the distance between the two parts.

Optimal results were obtained with a *trans, trans*-butadiene spacer which improved the binding affinity significantly ($K_D$=0.035 mM; $IC_{50}$= 75μM). Further studies showed that both classes of ligands bound to the same location on E2 DBD. Therefore, a single compound **84** was synthesized which incorporated the active substituents of each compound, both the butadiene spacer and the dichlorosubstituents. A filter binding essay revealed this new



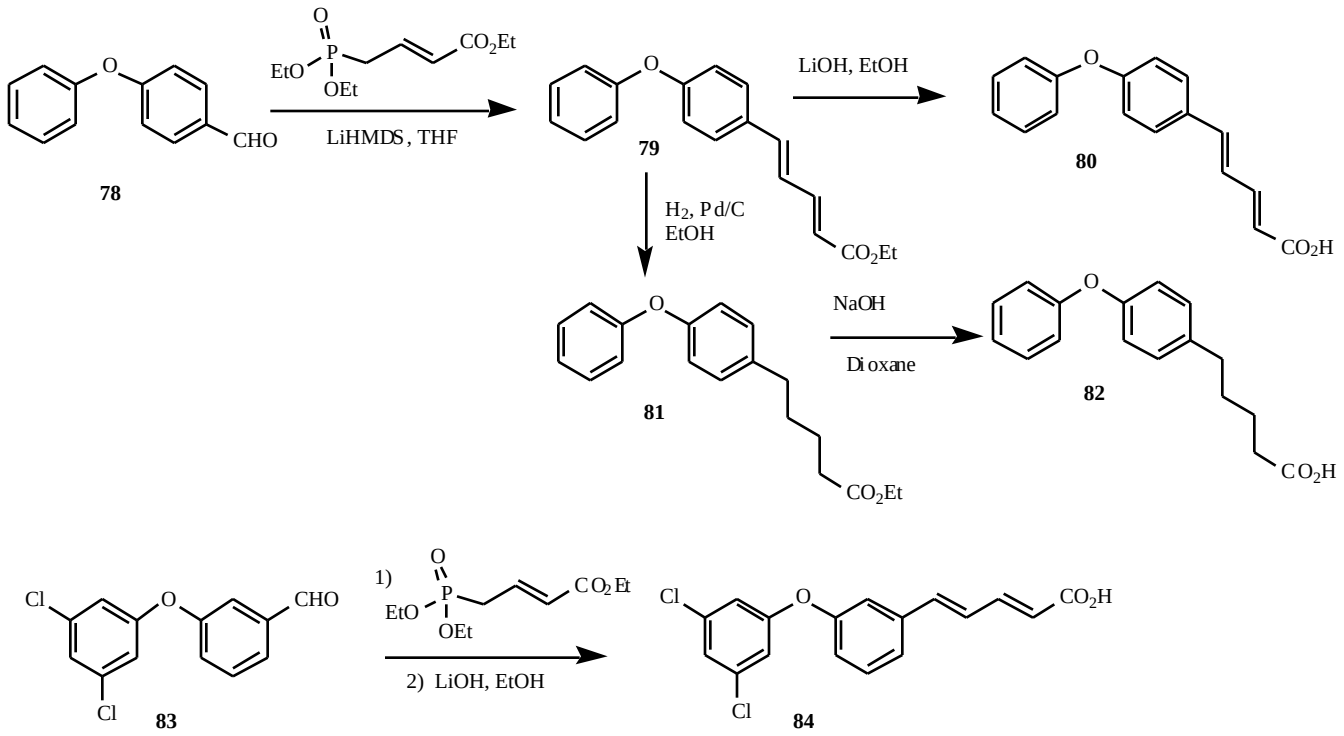

**76a/b** **77a-z**

**A**: X=Br (**76a**), Y= $B(OH)_2$, $Pd(PPh_3)_4$, 2M $Na_2CO_3$, DME/EtOH, reflux, 20h

**B**: X=$B(OC_3H_6)_2$ (**76b**); Y=Br, $Pd(PPh_3)_4$, 2M $Na_2CO_3$; DMF/$H_2O$, 80°C, 2d

**C**: X=$B(OC_3H_6)_2$ (**76b**); Y=OTf, $Pd(OAc)_2$/dppf, 2M $Na_2CO_3$, toluene/EtOH, reflux, 2d

**Scheme 9**. General synthesis of biphenyl carboxylates.




**Scheme 10**. Synthesis of biphenyl ether carboxylates **80**, **82**, and **84**.

compound to be the most potent of all with an $IC_{50}$ value of 10 μM (Fig. (**20**)).

As exemplified in the preceding three examples, Fesik and colleagues advocate SAR by NMR as a useful method for discovering high affinity ligands. They claim that the binding affinities of individual fragments are multiplied upon linkage; consequently, the binding affinities of individual fragments can be enhanced from millimolar to submicromolar affinities upon linking. Other advantages suggested by the authors include the ease of detection of small, weakly bound ligands using $^{15}$N-HSQC spectroscopy, the speed with which different binding site locations can be determined, and the limited synthetic

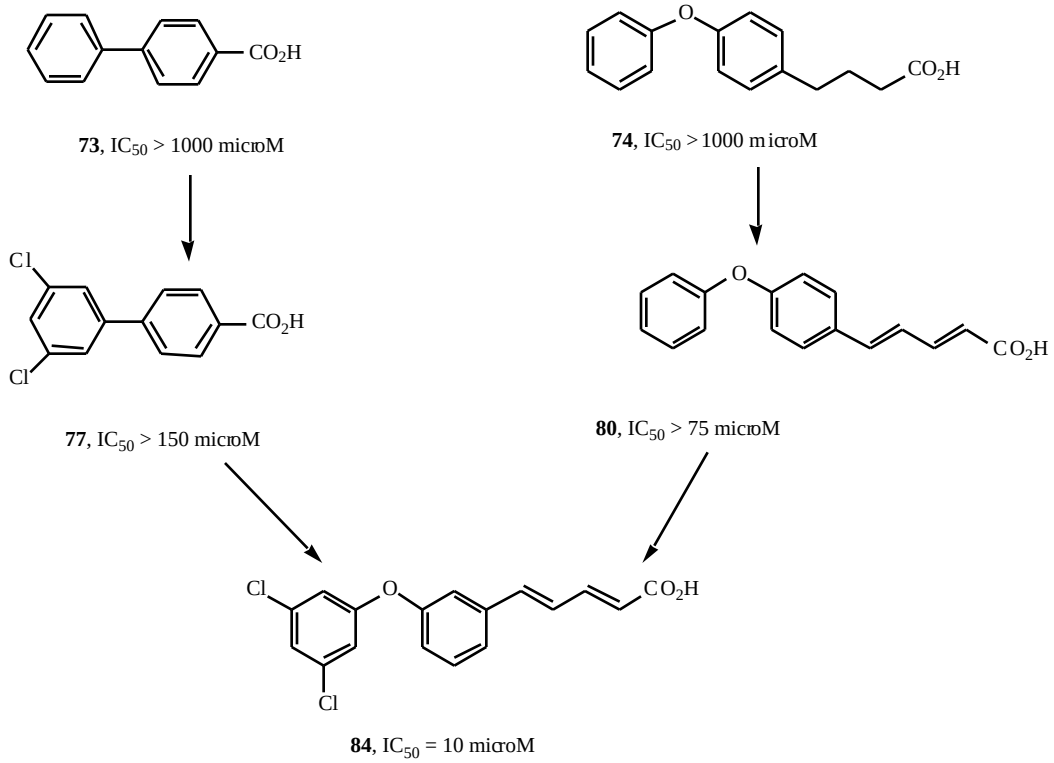
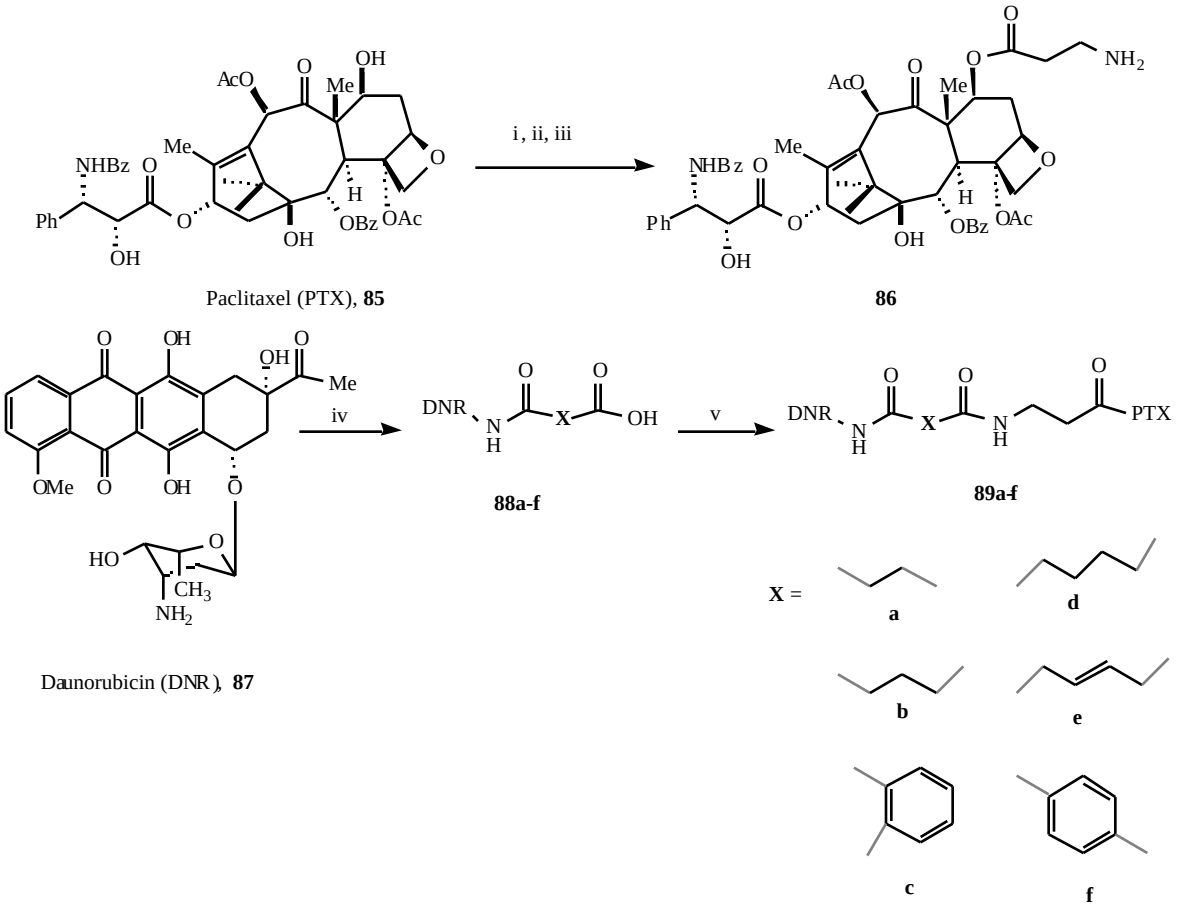



**Fig. (20)**. Summary of the discovery of a 10μM inhibitor of HPV-E2 DBD starting from millimolar leads identified using SAR by NMR.

chemistry required since the untethered ligands are optimized prior to linking. One disadvantage acknowledged by Fesik *et al.* is the fact that the SAR by NMR method is applicable only to biomolecules of molecular weight less than 30kD that can obtained in large quantities (≥ 200mg).

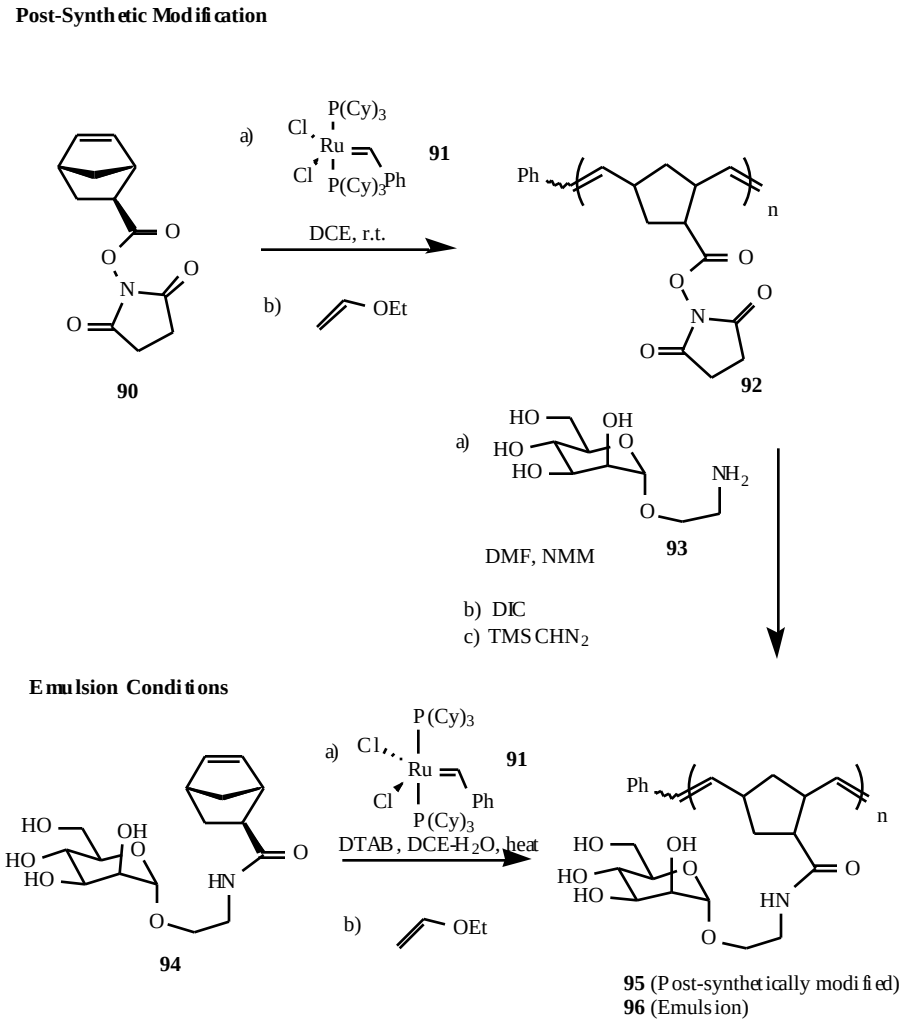
To add to the multitude of examples yet another novel case that exploits the idea of achieving increased affinity by multivalent binding, Wandless and colleagues have attempted to synthesize bifunctional polymers comprised of paclitaxel (PTX) (**85**) and daunorubicin (DNR) (**87**) [27]. These heterodimeric compounds could potentially interact with both microtubules and DNA at the metaphase-anaphase transition of mitosis, where the nuclear membrane is no longer present in human cells, thus increasing the activity over the parent monomoers based on the principles of polyvalency. The dimers were synthesized as outlined in Scheme 11. Based on data that indicate that bulky groups can be attached to the C7 hydroxyl group of PTX and that the sugar moiety of DNR can be selectively altered without a significant loss in biological activity of either compound, a range of linkers were designed to construct the dimers. Although the two compounds can be covalently linked without losing the ability to interact with their respective targets, cytotoxicity assays showed that the dimers were less potent than the parent monomers. The authors propose that increased steric bulk of the dimer compared to the individual compounds may interfere with the ability of each half of the dimer to bind to its target.

The advantages of multivalent interactions in improving binding affinity and enhancing signal transduction have been exploited not only in the study of protein-protein interactions, but also in the area of carbohydrate chemistry. Kiessling *et al.* have achieved significant success in studying the effects of multivalency on carbohydrate-protein binding interactions [28]. Ring opening metathesis polymerization (ROMP) has made possible the synthesis of large libraries of carbohydrate substituted polymers for such studies [29, 30]. Kiessling and colleagues claim that ROMP is advantageous as it can be used to create a vast array of polymers, that can be subsequently functionalized, using a single set of reaction and purification conditions. Also, with ROMP, the length of the polymer can be systematically varied by altering the monomer to catalyst ratio. Kiessling and co-workers used ROMP to generate a polymer backbone and then subsequently modified the backbone to generate a functionally diverse oligomer as outlined in scheme 12. This method, an alternative to the emulsion method in which the fully functionalized starting material is polymerized,




(i) Benzylchloroformate, pyridine, $CH_2Cl_2$, 65%; (ii) *N*-Cbz-β-alanine, DCC, DMAP, $CH_2Cl_2$, 95%; (iii) $H_2$, Pd/C, MeOH, 95%; (iv) Condition 1: succinic, glutaric or phthalic anhydride (5 equiv), 2,6-lutidine (2 equiv), MeOH, rt, overnight, 56-90%. Condition 2: terephthalic acid or *trans*-β-hydromuconic acid (2 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), DMF, rt, 30 min, 35-50%; Condition 3: adipic acid mono ethyl ester (2 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), DMF, rt, 30 min, then LiOH (3 equiv), THF:$H_2O$ (3:1 v/v), 40%; (v) 86 (0.9 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), THF, rt, 30 min, 40-70%.

**Scheme 11**. Synthesis of daunorubicin-paclitaxel dimers.




**Scheme 12**. Two Synthetic Roues Used to Generate Analogous Multivalent Mannose Displays

produced oligomers which were equally or more effective than those synthesized under emulsion conditions. ROMP reaction with the norbornene monomer **90** derivatized with an acyl succinimide produced the low molecular weight oligomers **92** after capping of the propogating alkylidene with ethyl vinyl ether. Acylation of a mannose derivative containing an amino terminating tether at the anomeric position gave the sugar derivatized oligomers **95**.

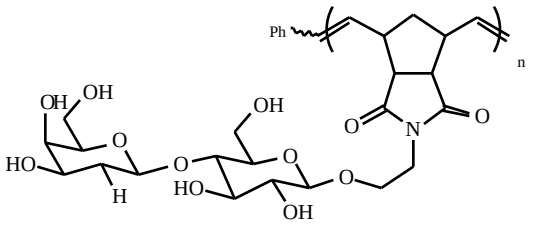
The mannose-substituted polymers were designed to interact with the lectin, concavalin A, a homotetramer that facilitates the agglutination of red blood cells by simultaneous interactions with mannose residues on the surfaces of adjacent cells. Experimental results indicated that the most potent inhibitors are able to span two saccharide binding sites on Con A [31]. These were produced with a 50:1 monomer to initiator ratio. Kiessling *et al.* report that the oligomers prepared by post-synthetic modification were slightly more active than those prepared under emulsion conditions, which is not surprising since these oligomers were found to be slightly longer and thus probably better able to span the binding sites on Con A.

Based on studies with Con A, Kiessling *et al*. have shown that chain length of the polymer greatly affects the affinity of the neoglycopolymers for Con A, with increasing chain length resulting in an exponential increase in binding affinity. A 100-fold enhancement relative to the monomer at DP=10 vs. a 2000-fold enhancement at DP=52 was observed. Kiessling and colleagues propose that both a chelation effect as well as a statistical effect, resulting from high local concentration of carbohydrate residues, are responsible for the dramatic increase in binding affinity to Con A with increasing polymer length.

The applicability of ROMP has been extended to the synthesis of neoglycopolymers to investigate the activity of L-selectin, a cell adhesion molecule that mediates the process of leukocyte rolling, an initial step in the inflammatory response [32]. The polymers, designed to present multiple copies of saccharide epitopes on an extended scaffold, were successful in promoting loss of L-selectin from the cell surface by protein shedding, the proteolytic release of a cell surface protein. The monovalent ligand was unable to induce this process. The authors suggest that ligand clustering is the reason for the enhanced activity.

A year later, Kiessling and colleagues investigated the mechanism of increased potency of multivalent ligands using the two lectins, galectin 3 (Gal-3) and *Erythrina corallodendrum* (EcorL) [33]. The receptor binding sites of these lectins are oriented on opposite faces. Using the lactose

neoglycopolymer **97** (Fig. (**21**)), studies were undertaken to observe the increase in binding elicited by multivalency.

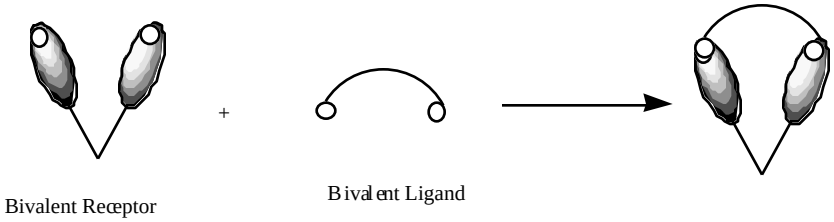



**97**

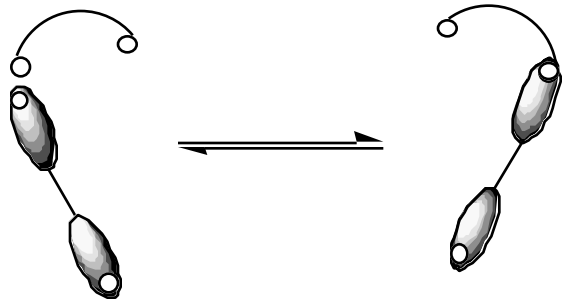
**Fig. (21)**. Structure of lactose neoglycopolymer.

Although binding affinity was enhanced in the presence of the polymer compared to monovalent lactose, the increase was not exponential as observed in previous studies with Con A. The authors propose that this is because of the difference in binding site orientation between the lectins and Con A (Fig. (**22**)). Unlike Con A , neither lectin is able to benefit from the chelate effect because their receptor binding sites are on different faces. As a result, the relatively small increase in binding affinity observed in the presence of the lactose neoglycopolymer is due to a statistical effect, a less potent effect than chelation. Con A, which has multiple binding sites on the same face, is able to benefit both from the chelation and high local concentration, hence the exponential increase in binding affinity. The authors claim that these results provide valuable information for choosing targets for multivalent ligand design.

To further understand the intricacies of multivalent binding interactions, Kiessling *et al*. undertook a study to determine the effect of distinct modulation of the number of biologically active epitopes presented by a multivalent ligand on the resultant biological response, in this case bacterial chemotaxis [34]. During chemotaxis in *E. coli*, specific receptors recognize chemo-attractants, such as galactose, and chemo-repellants at the bacterial plasma membrane. In the case of galactose-mediated signaling, galactose must bind to the soluble periplasmic




Binding Enhancement due to simultaneous spanning of two binding sites (the chelate effect).



Enhancement of binding due to increased local concentration of available ligand (the statistical effect).

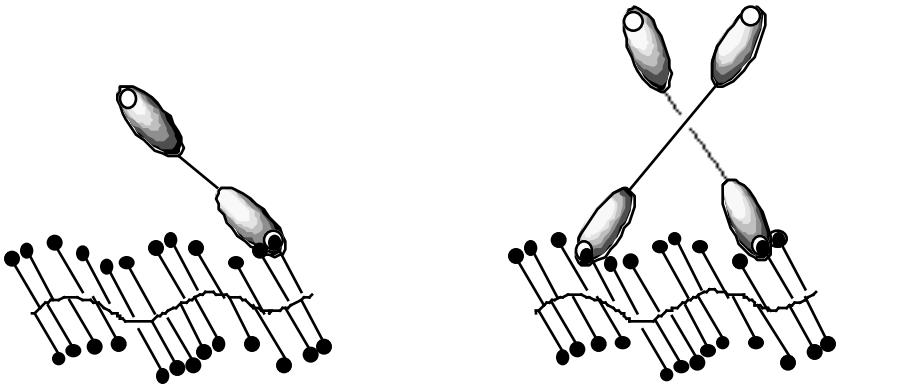



**Fig. (22)**. Mulitvalent modes of binding.

glucose/galactose binding protien (GGBP), which in turn, interacts with the galactose-sensing chemoreceptor, Trg. Formation of the galactose-GGBP-Trg complex initiates a signaling pathway that promotes a reversal of the direction of flagellar spin. This series of events results in the direction of the bacteria towards the nutrient. The authors claim that they can tune this chemotactic response of *E. coli* and *Bacillus subtilis* by altering the valency of the ligands that cause clustering of the bacterial chemoreceptors. This modulation of ligand valency influences chemotactic response by altering the extent of cell surface chemoreceptor clustering. The more binding sites available on the ligand, the greater the degree of receptor clustering. Kiessling *et al.* utilized ROMP to synthesize a range of galactose-bearing ligands of distinct length and varying valencies (Fig. (**23**)).

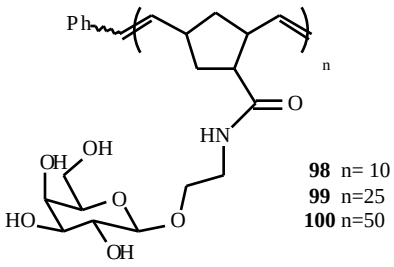



**Fig. (23)**. Structure of the ROMP derived multivalent arrays displaying galactose.

Results indicated that the longer oligomers with increased ligand valency were the most potent, inducing chemotaxis at lower concentrations. The galactose-residue concentration of maximum chemotaxis was 0.25 mM for oligomer **99** and 0.10mM for the longer **100**. Based on results of fluorescence microscopy experiments that visualized changes in receptor organization upon treatment with ligand, the ROMP-derived multivalent compounds were shown to induce receptor clustering. The experimental data indicated that the valency of the ligand influenced its ability to cluster the chemoreceptors, thereby eliciting a chemotactic response.

Various analytical methods have been explored for efficiency in monitoring changes in multivalent binding interactions. Swanson and Song have proposed a method of detection of toxins that takes advantage of the multivalent binding that occurs between bacteria toxins and oligosaccharide receptors on a host cell surface [35]. The general fluorescence transduction method is based on fluorescence quenching of ligands held in close proximity by mulivalent interactions. According to the authors, this scheme, which couples the distance-dependent self-quenching of fluorescence to the multivalent binding between toxins and receptors, is advantageous for several reasons including high chemical and functional stability and increased sensitivity. They propose, as the main advantage of coupling recognition and signal transduction, the amplification of specific versus non-specific binding events.

Swanson *et al.* designed a ganglioside, F-GM1 (**101**), using fluorescein as the transduction element, without altering the binding affinity of the toxin for the pentasaccharide moitey (Fig. (**24**)).

Data indicated that upon binding of cholera toxin (CT) to F-GM1, fluorescence decreased dramatically as a result of self-quenching. This self-quenching occurred because binding of CT to F-GM1 brought the fluorescein fluorophores into the required proximity, a distance of approximately 50Å. Addition of 2nM of CT in 2nM of F-GM1 resulted in 80% decrease in fluorescence activity.

Another method, described by Kiessling et al., for monitoring multivalent binding interactions is transmission electron microscopy (TEM) [36]. This method has been modified to allow the visualization of relatively small receptor-ligand complexes of densities lower than can generally be imaged by TEM. By appending colloidal gold to the complexes, the density is increased, thus enhancing the contrast of the receptors as observed by TEM. Kiessling et al demonstrated the effectiveness of the method by labeling Con A tetramers with biotin, a molecule with high affinity for the streptavadin-conjugated gold particles, and observing the interaction of Con A with a series of multivalent ligands. The number of gold particles incorporated into complexes was regarded as an indication of the ability of the ligands to bind multiple copies of Con A. Kiessling *et al.* report that the modified TEM allowed direct visualization of the complexes and is effective even for relatively weak receptor ligand interactions ($K_D$=1mM).

To demonstrate yet a different method, surface plasmon resonance, used to rapidly screen low molecular weight compounds for inhibition or protein-carbohydrate

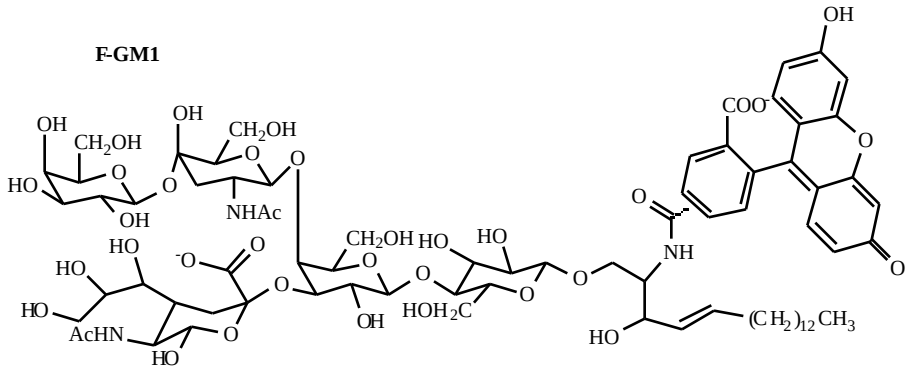



**Fig. (24)**. Structure of the fluorescein-labeled GM1, F-GM1.

interactions, Kiessling *et al.* synthesized a neoglycopolymer and noncovalently bound it to an optical sensor chip surface generating an artificial ligand-containing lipid bilayer [37]. The density of the saccharide ligands presented was controlled by combining the synthetic glycolipid with phosphatidylcholine in various molar ratios. Kiessling and colleagues were able to successfully and efficiently study both small, low-affinity monovalent as well as larger, high-affinity multivalent Con A ligands using solution competition studies that depended on the synthetic glycolipid surface.

## CONCLUSION

Recently, the important role of multivalent interactions in the regulation of key biological systems has become apparent. This insight into the modulation of biological function provides a conceptual starting point for the design of new therapeutic agents with enhanced potencies and selectivities. A variety of designs based on the multivalent model have been disclosed with very exciting biological activities. With such promising preliminary studies, the design of multivalent agents will continue as a useful strategy for the development of new therapeutic agents.

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