Design and Synthesis of a Pro-Drug of Vinblastine Targeted at Treatment of Prostate Cancer with Enhanced Efficacy and Reduced Systemic Toxicity

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Chemotherapy of prostate cancer with antimitotic agents such as vinblastine and doxorubicin is only marginally effective, due to dose-limiting systemic toxicity. Herein we report the development of peptidyl conjugate **5** of the cytotoxic agent vinblastine (**1**), along with the results of its in vitro and in vivo evaluation as a pro-drug targeted at prostate cancer cells. Prostate-derived tumors are known to produce significant amounts of prostate specific antigen (PSA), a serine protease with chymotrypsin-like properties. Earlier work in these laboratories established that an appropriately engineered peptidyl pro-drug will release active cytotoxic agent strictly within the microenvironment of the tumor tissue (Garsky, V. M., et al. *J. Med. Chem.* **2001**, *44*, 4216−4224). Conjugate **5**, which features an octapeptide segment attached by an ester linkage at the 4-position of vinblastine (**1**), undergoes rapid cleavage by PSA ($T_{1/2}$ = 12 min) between the Gln and Ser residues. In nude mouse xenograft studies, **5** reduced circulating PSA levels by 99% and tumor weight by 85% at a dose just below its MTD. By contrast, the putative end-point metabolite, the cytotoxic agent *des*-acetyl vinblastine (**1b**), was ineffective in reducing PSA levels and tumor burden at its maximum tolerated doses. Additional data from metabolism studies on **5** support the supervention of a novel in vivo processing mechanism, the spontaneous release of **1b** from a dipeptidyl intermediate driven by favorable diketopiperazine formation.

Introduction

Metastatic cancer of the prostate is associated with a high degree of morbidity and mortality and is predicted to have resulted in the death of up to 32000 men in the United States during the year 2001. As long as the cancer is confined to the prostate, it can be successfully controlled by surgery or radiation, but in metastatic disease, few options are available beyond androgen ablation,[1] the mainstay of treatment in the case of lymph node involvement or disseminated loci. Once tumor cells have become hormone-refractory, the standard cytotoxic agents are only marginally effective in slowing disease progression, although they do provide some degree of palliative relief. Current chemotherapeutic regimens, typically combining two or more agents, afford response rates in the range of only 20−30%.[2,3] With the utilization of vinblastine, doxorubicin, or similar antimitotic agents, efficacy is limited by significant systemic toxicity.

We and others[4,5,6] have endeavored to address the need for improved antitumor therapy by means of a number of pro-drug approaches, and a recent report from these laboratories details the development of a peptidyl conjugate of doxorubicin effective in an animal model against prostate tumor xenografts.[7] In this endeavor the enzymic properties of prostate specific antigen (PSA) have been exploited to convert an inactive pro-drug of doxorubicin into active cytotoxic agent at the site of the tumor. The modality employed features a doxorubicin-linked peptide sequence optimized for cleavage by PSA, a serine protease with chymotrypsin-like activity that is a member of the kallikrein gene family.[8] Within the prostate the apparent physiological role of PSA is to enhance the liquefaction of semen via its action on the semenogelins, a group of proteins found in seminal fluid. Prostate tumor cells are known to secrete significant amounts of PSA, a fact which forms the basis for the measurement of serum PSA levels in clinical screening for prostate cancer. Moreover, circulating PSA levels have been found to correlate well with the extent of malignancy, heightened levels typically signaling metastatic disease. Once in the systemic circulation, however, PSA lacks enzymatic activity due to complexation with α1-antichymotrypsin and α2-macroglobulin.[9,10]

The reported doxorubicin conjugate[7] exploits the proteolytic activity of PSA within prostate cancer tissue to facilitate release of cytotoxic agent selectively into the microenvironment of tumor cells. Since the activity of PSA is substantially reduced in the systemic circulation, high concentrations of cytotoxic agent localized at the tumor site can be realized. In the present work, the development of a peptidyl conjugate of the cytotoxic agent vinblastine (**1**) is detailed, wherein selective action against prostate tumor cells is postulated to occur by

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analogous means. Thus, a number of peptides selected for rapid cleavage by PSA were covalently linked to vinblastine to provide a series of peptide-vinblastine conjugates which were evaluated both in cell culture and in animal models of human prostate cancer.[11] On the basis of these studies, we selected 4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-Pro)-*des*-acetyl-vinblastine (**5**) for further investigation to assess its ability to inhibit prostate cancer cell proliferation and tumorigenesis.

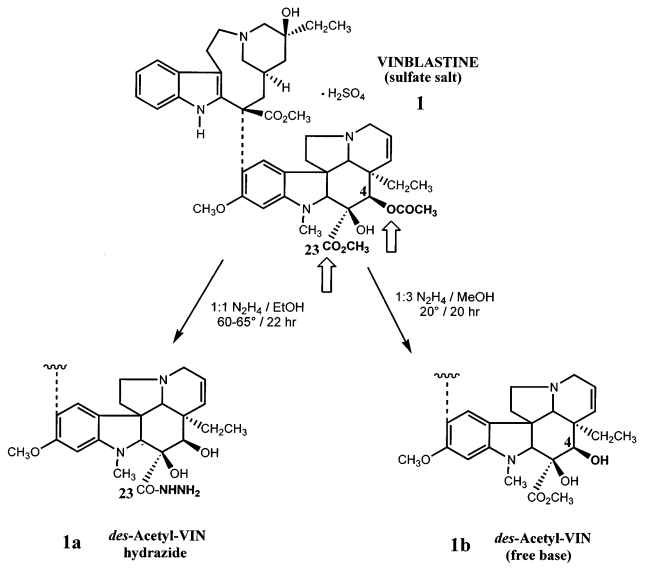
Design and Synthesis

During the initial phases of this effort, we were guided by results from the prior work with doxorubicin, which established features critical for peptide-based targeted delivery of cytotoxic agents. Thus, the basic approach was to identify a functionality on vinblastine appropriate for covalent linkage of a peptide unit, wherein the resulting conjugate would possess no intrinsic cytotoxic activity. Additionally, the peptide segment would have to be sufficiently rapidly cleaved by PSA if active cytotoxic entity was to be generated efficiently and in high yield. Further down the line, a high degree of selectivity for PSA-secreting cells versus a suitable control cell line (PSA nonsecreting), minimal susceptibility to nonspecific enzyme cleavage in serum, and efficacy in a mouse tumor model for prostate cancer would ultimately be required.

As we considered the opportunities and challenges in linkage of a peptide to vinblastine, we recognized several important differences with respect to doxorubicin. For one, the vinblastine molecule afforded, based upon our assessment of the literature,[12,13] at least two relatively accessible sites for derivatization (see structure **1**): (1) the carbomethoxyl group at position C23, by virtue of its convertibility to the corresponding reactive carboxylic acid functionality; (2) the acetoxyl group at position C4, by virtue of its convertibility to the corresponding reactive hydroxyl functionality. Second, the vinblastine molecule was known to be chemically stable over a much wider range of pH and media than doxorubicin, affording a greater choice of methods and conditions in chemical manipulations. Third, the solubility properties of the vinblastine family generally conform to those of amine bases, by virtue of the two basic nitrogens, resulting in markedly increased solubilities of derivatives upon increasing acidity, particularly below pH 7.

As was the case with respect to doxorubicin, our immediate goal in the present work became to determine whether one or the other of the aforementioned reactive sites on vinblastine, when conjugated with a peptide fragment, would yield a compound of little or no cytotoxic activity in cell culture and whether, when the peptide in question was readily cleavable by PSA, the end product after such cleavage would exhibit the appropriate cytotoxic activity profile. Proceeding on the basis of the extensive peptide substrate database compiled as the optimal sequence for conjugation to doxorubicin was engineered,[7] we realized that a number of consistent residue preferences were already recognized. In particular, we were impelled to retain the −Gln−Ser− motif at the scissile site ($P_1 \sim P_1'$), unique among proteases, for which a strong preference vis-à-vis PSA had been determined over a range of substrates. Likewise, we expected to be able to utilize the findings of previous studies of residue replacements throughout the cleavage site region to choose a number of likely sequences as starting points for our conjugate design.

Scheme 1. Vinblastine Derivatization



It was the chemical manipulation of the vinblastine nucleus that occupied the major portion of our attention as we initiated our synthetic planning. As noted above, prior investigations have utilized mainly two positions on the vinblastine molecule for covalent linkage of various aminoacyl or peptidyl units, in efforts studying a variety of approaches to enhancing tumor selectivity and therapeutic index of agents within the vinblastine family.[14] Scheme 1 depicts the chemical transformations involved in exposing the functionalities at positions C4 and C23, in readiness for subsequent reactions. Thus, heating vinblastine sulfate (**1**) with 50% hydrazine in ethanol for 1 day results in removal of the acetyl group at C4 and specific formation of the C23 hydrazide **1a** in high yield. However, if the reaction is conducted at room temperature with 25% hydrazine, the C4 acetyl moiety is still completely removed, but the C23 ester remains untouched to the extent of 95%, affording *des*-acetyl vinblastine (dAc-VIN, **1b**). Compound **1a** is ideally disposed for specific derivatization via activation as the acyl azide, followed by amidation, a process which has been applied to prepare, variously, amidoalkyl esters,[15] amides from alpha amino esters,[16] as well as immunoconjugates of monoclonal antibodies.[17] In a similar fashion, the free C4 hydroxyl group of compound **1b** has served as precursor to immunoconjugate succinate esters[18] and a series of N-maleoylamidoalkyl esters.[19−21]

In the present investigation, we first focused on the preparation of amides at position C23, specifically employing reaction of the acyl azide, generated in situ, with monoprotected diamines to afford, after blocking group removal, an amide with a straight-chain alkylamino function, appropriate for conjugation with peptide substrates of PSA. This endeavor led to an early series of conjugates with peptides linked to *des*-acetyl vinblastine via alkyl and cycloalkyl diamides at C23, which, despite promising results in in vitro bioassays, failed to exhibit suitable efficacy in vivo (data not shown).

Table 1. Biological Data

		in vitro				in vivo			
			EC_{50} (μM)						
	vinblastine-conjugate structure	$T_{1/2}$[a] (min)	LNCaP[b]	Colo320[c]	T47D[d]	% PSA[e] reduction	% tumor[e] wt reduc	dose (μmol/kg)	MTD[f] (μmol/kg)
1	vinblastine (VIN)		0.5	–[g]	–[g]	–	–	–	–
1b	*Des*-acetyl vinblastine (dAc-VIN)	–	0.1	0.2	0.15	14	16	0.26	0.26
2a	4-O-(**Leu**)-dAc-VIN	–	4.0	6.0	–	–	–	–	–
2b	4-O-(**Gly**)-dAc-VIN	–	0.3	1.8	–	–	–	–	–
2c	4-O-(**Pro**)-dAc-VIN	–	1.0	3.0	1.6	33	60	4.6	4.6
3a	4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-**Leu**)-dAc-VIN	35	40	87	–	–	–	–	–
3b	4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-**Gly**)-dAc-VIN	30	9.3	13.5	–	–	–	–	–
3c	4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-**Pro**)-dAc-VIN	8% (1 h)	48	84	–	–	–	–	–
5	4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-**Pro**)-dAc-VIN	**12**	1.6	14	>50	99	85	15.3	21.4
6	4-O-(Ac-Hyp-Ser-Ser-Chg-DGln-DSer-Ser-**Pro**)-dAcVIN	no clvg.	16	48		54[h]	64[h]	12.3	ND

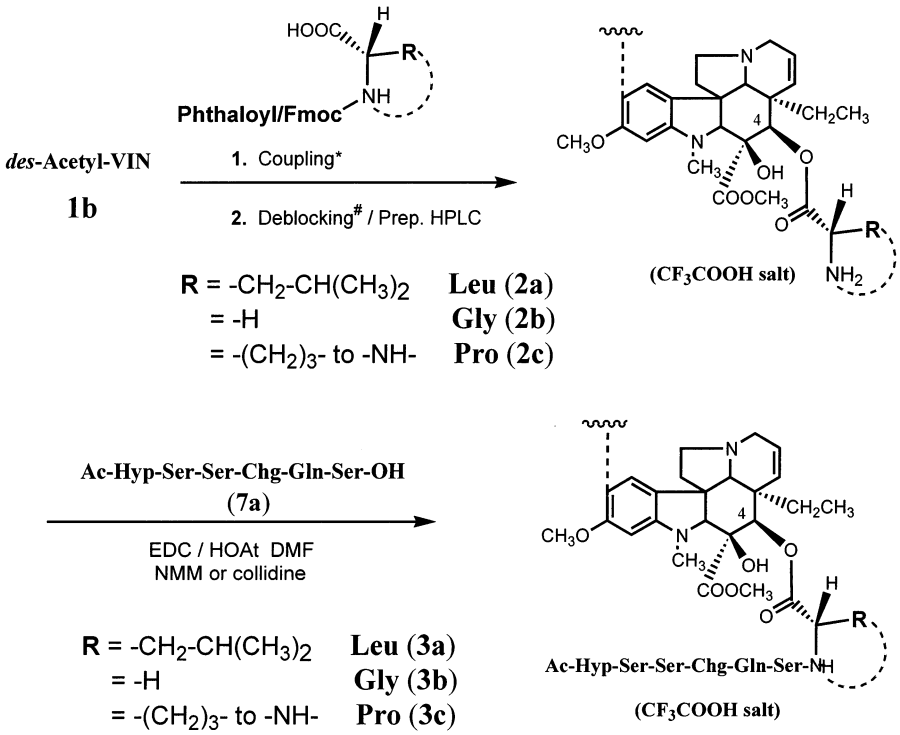
[a] $T_{1/2}$ = time required for PSA to hydrolyze 50% of compound at a molar ratio of 1:100, respectively. [b] LNCaP, PSA secreting human prostate cancer cell line. [c] Colo320, PSA nonsecreting human colorectal carcinoma cell line. [d] T47D, PSA nonsecreting human breast ductal carcinoma cell line. [e] Values in percent relative to vehicle control following intraperitoneal injection (i.p.) of conjugate (dose) for 5 consecutive days to nude mice bearing LNCaP tumor xenografts. *P* (values): **1a** dAcVIN (0.549); **2c** (0.400); **5** (0.001); **6** (0.506). [f] MTD, defined as maximum nonlethal drug dose which could be administered (i.p.) to nontumor bearing nude mice for 5 consecutive days. P (values): **1a** dAcVIN (0.595); **2c** (0.226); **5** (0.005); **6** (0.181). [g] Value determined in human bladder carcinoma cell line (T24) as ≤0.08 μM. [h] Percent increase.

In a parallel study, we targeted the secondary C4 hydroxyl group for derivatization in the form of C-terminal peptide esters, which would serve as precursors to final processing product dAc-VIN (**1b**) in vivo. Initial comparison of dAc-VIN with vinblastine (**1**) in these laboratories established the essential equivalency of both compounds with respect to toxicity in cell culture and pharmacokinetic behavior in vivo (Table 1). This finding is consistent with previous comparisons among several members of the vinblastine family,[12] making *des*-acetyl vinblastine eminently suitable as the end point cytotoxic agent. Significantly, *des*-acetyl vinblastine (**1b**) is the major metabolite of vinblastine (**1**) in humans,[22] thus in effect the pharmacological equivalent of vinblastine. Cell-based assay protocols employed a prostate carcinoma-derived PSA-secreting cell line (LNCaP), with several types of non-PSA-secreting tumor-derived cell lines serving as controls, to compare cytotoxicity with respect to dependence or nondependence upon PSA (see Biological Results section).

A more immediate concern was the potential susceptibility of any 4-position conjugate to premature cleavage in vivo through the action of nonspecific esterases, although such cleavage had not been reported in earlier work.[20] Accordingly, it was decided at the outset to assess several 4-O-(α-aminoacyl) derivatives to determine the effect, if any, of esterase activity in bioassays. It quickly became clear that acylation of the C4 hydroxyl function of **1b** was in fact subject to specific difficulties associated with crowding due to constraints inherent in the pentacyclic ring system and to direct steric hindrance from multiple neighboring groups. In this connection, the literature[19] offered some guidance, insofar as amino acid esters at C4 were shown to be accessible via anhydride intermediates when the protected amine had no free hydrogens. In our hands, under a number of conditions including mixed anhydride protocols and DMAP-catalyzed O-acylation,[23] Boc-leucine underwent only minimal reaction with the hydroxyl group, but the corresponding N-phthaloyl derivative afforded a modest yield of the acylated product upon mixed anhydride formation with isobutyl chloroformate in the presence of triethylamine, as shown in Scheme 2. Phthaloyl removal under standard conditions using hydrazine gave 4-O-Leu-*des*-acetyl vinblastine (4-O-(Leu)-dAc-VIN) (**2a**). In a similar manner N-phthaloylglycine, upon mixed anhydride esterification followed by phthaloyl removal, afforded 4-O-(Gly)-dAc-VIN (**2b**). Fmoc-proline, however, failed to react as the mixed anhydride and required activation as the acyl chloride, prior to reaction with dAc-VIN **1b** in pyridine under rigorously anhydrous conditions. The standard treatment with piperidine in DMF afforded 4-O-(Pro)-dAc-VIN (**2c**). These findings made it evident that position C4 peptide conjugates would best be prepared from the single amino acyl derivatives, through coupling with the appropriate peptidyl PSA substrate segment, since direct coupling of a peptide with the 4-hydroxyl group would be difficult or impossible to accomplish, in light of the above constraints.

At this juncture it remained for us to choose the particular substrate sequence to enable the most expedient assessment of the 4-position ester pro-drug modality. It happened that from the extensive database built up during the doxorubicin effort,[7] there was a choice of a number of peptides having suitable PSA substrate properties. In particular, the sequence represented by peptidyl acid Ac-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH was the optimal substrate fragment incorporated into doxorubicin. Earlier studies of vinblastine conjugates had shown the Ala → Ser replacement to improve solubility. Thus, in the first examples of prototype ester conjugates, we decided to couple the peptide Ac-Hyp-Ser-Ser-Chg-Gln-Ser-OH (**7a**) with the 4-O-aminoacylated residues Leu, Gly, and Pro, respectively, on vinblastine. The peptide was prepared by standard solid-phase synthesis using an Fmoc/*tert*-butyl-based strategy starting from protected serine attached to hydroxymethylphenoxy (Wang-type HMP) resin. Cleavage of the peptide from the solid support and removal of the side chain protect-

Scheme 2. Acylation at the 4-Hydroxyl



Reagents and conditions: *1. N-phthaloyl deriv. / mixed anhydride, EtOAc-24 h (**2a**,**2b**)
N-Fmoc deriv. / acid chloride, pyridine-24 h (**2c**)
#2. N-phthaloyl deriv. / 2 equiv hydrazine, CH_3OH (**2a**,**2b**)
N-Fmoc deriv. / 30% piperidine, DMF (**2c**)

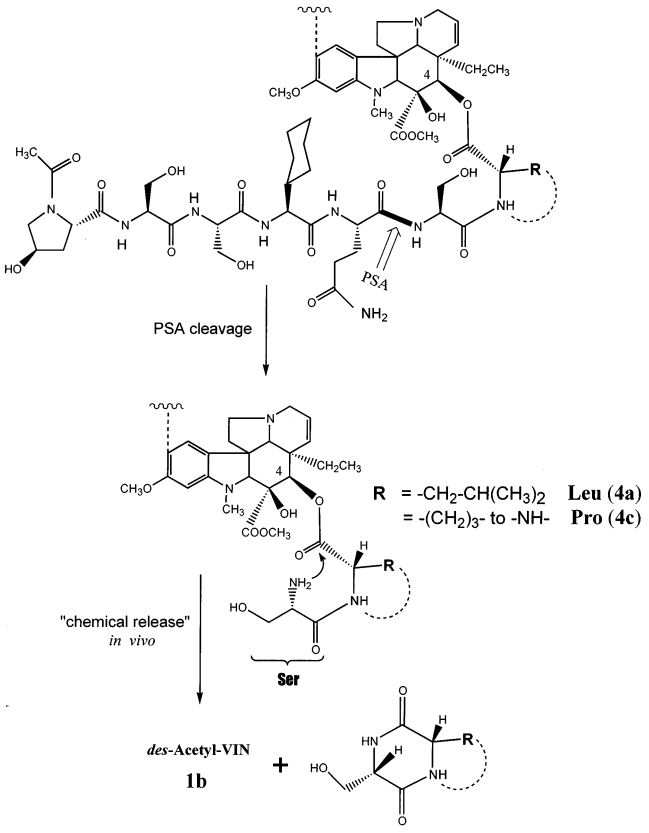
ing groups were effected using TFA-H_2O-TIPS (95:2.5:2.5 v/v). In the above instances, the peptides were used without purification in coupling of the C-terminal carboxyl group to the amino acid in question. A standardized set of coupling conditions using carbodiimide with N-hydroxyazatriazole (HOAt) as additive (see Experimental Section) afforded samples of the respective conjugates **3a**, **3b**, and **3c** in reasonable yield (Scheme 2). Final purification was carried out by means of reverse-phase high-performance liquid chromatography (RP-HPLC) with a Waters C18 Delta Pak (15 μm, 100A) column. Identity and purity of the products were determined by analytical HPLC, amino acid analysis, and FAB mass spectrometry. Conjugates containing other peptidyl sequences were prepared in the same manner (see below and Experimental Section for details).

Biological Results

Cytotoxic activities of the peptide-vinblastine conjugates were evaluated in selected cell culture media for comparison with both vinblastine and *des*-acetyl vinblastine. Details of the experimental biology have been reported[24] (also Table 1 footnotes). The PSA-producing LNCaP cell line, employed in the work with doxorubicin,[5] served equally well in the current series, but the prostate-derived non PSA-producing DuPRO control line proved to be insensitive to vinblastine. After examination of a number of alternatives, we selected a colorectal tumor-derived cell line, Colo320, for most applications and a breast carcinoma-derived cell line, T47D, in situations where we felt a greater degree of consistency was required. Table 1 depicts the results of assays on the reference cytotoxic agents, as well as the aminoacyl and peptidyl conjugates described above. EC_{50} values represent the compound dose required to kill 50% of the tumor cells. *des*-Acetyl vinblastine represents the endpoint active cytotoxic species generated from the peptide-vinblastine conjugates following PSA-mediated hydrolysis at the Gln-Ser (P_1—P_1') bond with subsequent processing of the intermediate 4-O-(Ser-Aaa)-dAc-VIN within the enzyme-rich cytosol of the prostate cancer cells (data not shown). Rates of hydrolysis by PSA (Table 1) are presented in terms of time required for 50% cleavage ($T_{1/2}$).

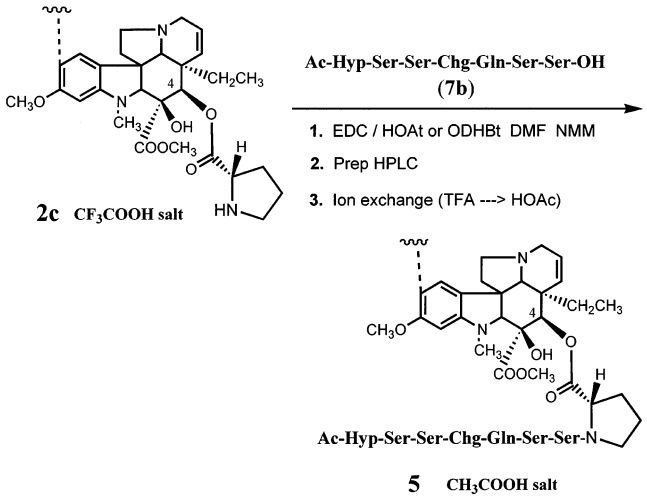
An earlier report on a series of 4-O-aminoacylated derivatives of vinblastine evaluated in a murine leukemia model showed some variation in retained cytotoxicity, although generally reduced toxic effect was associated with nonpolar substituents.[20] In a recent report from these laboratories,[24] the ester 4-O-(Pro)-dAc-VIN (**2c**) was described as cytotoxic in a number of cell lines, including the PSA-secreting LNCaP line, and as comparable in toxicity to VIN and dAc-VIN. In the current work, the Leu and Gly esters, **2a** and **2b**, respectively, exhibited cytotoxicity equivalent in all cell lines within experimental error, as shown in Table 1. Thus, we could not be sure to what extent esterase cleavage to give dAc-VIN might be responsible for the observed toxicity of these esters.

Scheme 3. Proposed Chemical Release Mechanism



The processing pathways became clearer when we explored the corresponding peptide conjugates. The data showed that compounds **3a** and **3b** had $T_{1/2}$ of 35 and 30 min, respectively, comparable to rates associated with tumor-selective conjugates in the doxorubicin series.[7] However, compound **3a** had only marginal activity in LNCaP cell culture, slightly better than in control cells, strongly suggesting that processing beyond the PSA cleavage point was failing to occur. Compound **3b** was modestly active in the LNCaP system, but in a separate experiment (data not shown) this conjugate, in contrast to conjugates **3a** and **3c**, was demonstrated to undergo significant hydrolysis in various cell culture media to give dAc-VIN, thus explaining its observed moderate degree of potency against non PSA-secreting cells. This instability was consistent with the erratic results on repeated cell-based assay, indicative of variable esterase-mediated cleavage upon assay in cell culture. Since esters **3a** and **3c** were found to be stable in cell-based media, these findings taken together served to define the limits of the role of esterase cleavage in generating end-point cytotoxic entity dAc-VIN (**1b**) from 4-position esters. However, it was also evident that stepwise proteolytic processing was relatively unfavorable in conjugates **3a** and **3b**. The low rate of PSA cleavage for the Pro conjugate **3c** served to further circumscribe our options, save for the fact that in this case an alternative pathway for generation of dAc-VIN made itself evident—namely, the possibility of spontaneous generation of dAc-VIN from the immediate cleavage product 4-O-(Ser-Pro)-dAc-VIN (**4c**). This hypothetical conversion, illustrated in Scheme 3, exploits the generally recognized propensity for dipeptide esters

Scheme 4. Synthesis of Conjugate **5**



to form diketopiperazines,[25,26] particularly if the C-terminal residue is a secondary amino acid.[27,28]

It was immediately evident that conjugate **3c** was inappropriate for testing this idea, since the low rate of cleavage was failing to generate sufficient intermediate within the time frame of the cell-based assay. Fortunately, earlier data from PSA cleavage of peptide sequences led us to expect that the introduction of an additional Ser residue into conjugate **3c** within the prime region would very likely increase the cleavage rate. One would then hope for nonspecific aminopeptidase processing of the thus-formed tripeptide species to generate the requisite dipeptide intermediate. As a means to test the rationale of this approach, putative intermediate **4c** (Scheme 3) was independently synthesized from 4-O-(Pro)-dAc-VIN (**2c**) by coupling it with Boc-Ser(tBu)-OH and removal of the Ser protecting groups with TFA; likewise as a control, the analogous species 4-O-(Ser-Leu)-dAc-VIN **4a** derived from conjugate **3a** was also prepared from 4-O-(Leu)-dAc-VIN (**2a**). In a strictly chemical environment (buffered pH 7.5, 25°), compound **4c** was converted to dAc-VIN, along with presumably cyclo-(Ser-Pro) with a $T_{1/2} \sim 6$ h, whereas compound **4a** exhibited barely detectable cleavage over 24 h (see Experimental section). This result confirmed the rate-enhancing effect of the secondary prolyl versus the primary leucyl residue in said context and led us to synthesize the octapeptide conjugate **5**. This conjugate was prepared by coupling of the heptapeptide Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-OH (**7b**) with the 4-O-prolyl ester of dAc-VIN **2c**, followed by purification by preparative RP-HPLC (Scheme 4). As the data in Table 1 show, conjugate **5** proved to be an exceptionally good substrate for PSA, with its $T_{1/2}$ of 12 min comparable to the best substrates to date. In cell culture, selectivity for PSA-secreting (LNCaP) versus both non-PSA-secreting cell lines was demonstrated. Thus, the EC_{50} for LNCaP cells, which secrete PSA, was 1.6 μM, as compared with an EC_{50} for T47D (the human breast ductal cell carcinoma line) of >50 μM and an EC_{50} for Colo320 (the human colorectal adenocarcinoma line) of 14 μM (Table 1). Significantly, conjugate **5** was shown to be entirely stable in human plasma over a period of 6 h. The observed sensitivity of the Colo320 line could result from some degree of tumor esterase-mediated cleavage between the C-terminal proline and the vin-

blastine nucleus. By contrast, neither dAc-VIN (**1b**) nor its prolyl ester **2c** exhibit more than marginal selectivity across the 3 cell lines. Thus, the results with compound **5** provided unequivocal evidence for our ability to target vinblastine in vitro to PSA-secreting human cancer cells through conjugation with the appropriate peptide substrate.

The critical objective of our effort was to demonstrate efficacy against PSA-secreting tumors in vivo. The antitumor activity of conjugate **5**, as well as that of 4-O-(Pro)-dAc-VIN (**2c**) and dAc-VIN (**1b**), was determined by assessing the ability of each compound to suppress the growth of human prostate cancer cell (LNCaP) xenografts in nude mice over the course of six weeks. Serum PSA levels, tumor weights, and whole animal weights were measured at the completion of the study. Table 1 also shows the percent PSA reduction in the treated animals versus vehicle controls following the planned protocol. Tumor weight loss correlates directly with reduction in PSA levels. In brief, the conjugate **5** at a dose just below its MTD (15.3 μmol/kg) effected a 99% reduction in average circulating PSA levels and an 85% reduction in tumor weight, compared with non-statistically significant changes of the same parameters when dAc-VIN **1b** and its 4-O-prolyl ester **2c** were administered. In the above protocol with conjugate **5**, the treated animals experienced a statistically insignificant 2% average total weight loss. The experimental biology has been reported in detail in a recently submitted publication.[24] (see also footnotes, Table 1)

The property of serving as a substrate for PSA is essential for vinblastine conjugate **5** to possess antitumor activity if it functions via the proposed biological mechanism underlying this concept. To be more convinced of the role of peptide as substrate, we prepared a conjugate that would not be a good substrate for PSA to subject to in vivo bioassay for antitumor activity. By incorporating the D-isoforms of Gln at P_1 and Ser at P_1', respectively, the corresponding conjugate 4-O-(Ac-Hyp-Ser-Ser-Chg-D-Gln-D-Ser-Ser-Pro)-dAc-VIN (**6**) was designed expressly to not be a substrate for PSA. As expected, **6** failed to show any evidence of cleavage on exposure to PSA for up to 24 h (Table 1). Moreover, when tested in the tumor xenograft model, compound **6** was no more effective than dAc-VIN (**1b**) against PSA-secreting tumors.

Additionally, realizing that optimal effectiveness may depend on the participation of a mechanism for spontaneous chemical generation of dAc-VIN (**1b**) from its 4-O-(Ser-Pro) ester **4c** driven by diketopiperazine formation (Scheme 3), we sought evidence that our proposed post-cleavage processing mechanism might be supervening over the expected stepwise proteolytic events. Such evidence was strongly suggestive in the findings from pharmacokinetic studies comparing the in vivo disposition of conjugate **5** with that of 4-O-(Pro)-dAc-VIN (**2c**), the requisite intermediate in stepwise processing. After i.v. administration to nude mice, the fraction of the total dose converted to cytotoxic end-point dAc-VIN (**1b**) was 43% from compound **5**, but only 8% from **2c**, suggesting the existence of a mechanism alternative to the stepwise conversion. Further support for such a mechanism was provided upon dosing with the potential metabolite intermediate 4-O-(Ser-Pro)-dAc-VIN (**4c**), of which the fraction metabolized to dAc-VIN (**1b**) was 51% (data not shown), demonstrating the relative efficiency in vivo of the direct conversion pathway predicted from the chemical studies.

On the basis of our in vitro and in vivo biological studies, conjugate **5** emerged with an optimal profile of pharmacological properties. Administration of this compound at the maximally tolerated dose (MTD) in the nude mouse model of prostate cancer afforded concentrations of cytotoxic agent at the site of the tumor 15-fold higher than if using the agent *des*-acetyl vinblastine at its MTD. In the same studies, circulating PSA levels were reduced by nearly 100% and weight of tumor by up to 90%, while total weight loss was limited to <10%. Neither dAc-VIN (**1b**) nor its 4-O-prolyl derivative **2c** had any discernible effect on PSA levels and tumor burden at their maximum tolerated doses.

Summary and Conclusions

The initial steps in designing a potentially clinically useful pro-drug of vinblastine have been guided by the successful earlier development in these laboratories of a pro-drug form of doxorubicin.[7] Thus, it has been demonstrated that appropriate derivatization of vinblastine greatly attenuates cytotoxic activity and that conjugates incorporating optimal peptide substrates of PSA are efficiently cleaved in cell culture such that the active cytotoxic entity, dAc-VIN (**1b**), is ultimately produced. Synthesis of the peptide-vinblastine conjugates employs a combination of peptide assembly on solid support and manipulation of the vinblastine nucleus using solution chemistry. Systematic modification of the peptide sequence has afforded conjugate **5**, which exhibits a rapid rate of PSA-mediated cleavage, selectivity for effect on a PSA-secreting tumor cell line, and efficacy in a nude mouse model employing xenografts of PSA-secreting human LNCaP prostate cancer cells. With respect to a range of features, compound **5** has been found to have the most favorable profile of physical and biological properties.

Compound **5** is digested by human PSA with a $T_{1/2}$ of 12 min while resisting attack by human proteases or other hydrolytic enzymes in whole blood or plasma (data not shown). The compound also exhibits significant selectivity for killing PSA-producing prostate cancer cells in cell culture versus non PSA-producing cancer cells from two cell lines, Colo320 derived from colorectal tumor tissue and T47D derived from breast carcinoma tissue (Table 1). In nude mouse xenograft studies using PSA-secreting implanted human prostate cancer cells from two PSA-secreting prostate tumor tissue lines, LNCaP (Table 1) and CWR22, [24] the vinblastine conjugate exhibits dramatically improved antitumor activity in comparison with dAc-VIN (**1b**), as measured by circulating PSA levels and tumor weights.[7] Moreover, conjugate **6**, a peptide-vinblastine conjugate that cannot be cleaved by PSA, evaluated under the same protocols, exhibits no activity against LNCaP-derived tumors, being no more effective than free dAc-VIN (**1b**).

The peptide−vinblastine conjugate **5** is intended for intravenous administration to treat hormone-refractory prostate cancer. It constitutes a pro-drug targeted at prostate tissue which is minimally toxic while in the free circulation, but which is activated by proteolytic

cleavage and subsequent processing to its cytotoxic end point metabolite *des*-acetyl vinblastine. The initial step in this activation is carried out by the serine protease PSA, an enzyme expressed in prostate epithelial tissue but not found at significant levels in other tissue. Subsequent processing via cellular aminopeptidases proceeds within the same cellular compartment, supplemented and possibly supplanted by spontaneous chemical conversion of the hypothetical dipeptidyl-vinblastine metabolite **4c** to dAc-VIN (**1b**), driven by formation of the diketopiperazine, *cyclo*-(Ser-Pro). As noted earlier, complexation of PSA with plasma proteins results in marked attenuation of its enzymatic activity once it enters the general circulation, so the peptide conjugate will not be cleaved by PSA except within the microenvironment of the cancer tissue. Thus, in theory peptide−vinblastine conjugate **5** should circulate freely in the body and be preferentially activated by PSA only at sites of prostate-derived tumor foci. Since the release of cytotoxic agent is compartment-specific, treatment with conjugate should constitute better therapy for disseminated prostate cancer than treatment with free vinblastine, with no enhanced toxicities. The data in support of highly tumor-selective delivery of dAc-VIN (**1b**) by the conjugate **5** in a nude mouse xenograft model of human prostate cancer have been discussed in detail.[24] Moreover, information supplied by preclinical studies,[24] consistent with pharmacological data from mouse tumor xenograft studies (see above), suggest that one may be able to effect delivery of upward of 10-fold more of the agent *des*-acetyl vinblastine via conjugate **5** to man on a molar basis, as compared with administration of vinblastine itself.

Experimental Section

Abbreviations. Abbreviations for common amino acids are in accordance with recommendations of IUPAC. Other abbreviations: t-Bu, *tert*-butyl; Trt, trityl; Chg, L-cyclohexylglycyl; Hyp, *trans*-4-hydroxy-L-prolyl; Ac, acetyl; Fmoc, fluorenylmethoxycarbonyl; NMP, *N*-methyl-2-pyrrolidinone; DMF, dimethylformamide; THF, tetrahydrofuran; NMM, *N*-methylmorpholine; DCC, dicyclohexylcarbodiimide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; HOAt, 1-Hydroxy-7-azabenzotriazole; ODHBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DMAP, 4-(dimethylamino)pyridine; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

General. Procedures implemented to ensure safe handling of vinblastine and its derivatives conformed to guidelines set forth by the Department of Safety and Industrial Hygiene, Merck and Co., Inc., specifying the manipulation, containment, and signposting regarding materials designated as constituting a PB-ECL P4 hazard level.

Conditions for analytical RP-HPLC were as follows: column no. 218TP5415 Vydac 4.6 mm i.d. X 15 cm C18 column, flow rate 1.5 mL/min at 214 and 280 nm. Mobile phases: $A = 0.1\%$ TFA/H_2O, $B = 0.1\%$ TFA/acetonitrile. System A: Gradient (95%A → 50%A) over 45 min. System B: Gradient (95%A → 5%A) over 45 min. System C: Gradient (85%A → 65%A) over 30 min.

Mass measurements of the compounds were carried out on one of the following three instruments: VG Autospec-Q by use of electrospray (ES) or fast atom bombardment (FAB) ionization; VG ZAB-HF by use of FAB; or the Bruker BioApex 7T FT/ICR/MS by use of ES. The *m*/*z* values are reported as $[M+H]^+$.

$T_{1/2}$ Determination: Time to 50% Cleavage of Peptide-Vinblastine Conjugates 3a, 3b, 3c, 5, and 6 by Purified PSA. Peptide conjugates were mixed with PSA at a molar ratio of 100:1, respectively, in 50 mM TRIS·HCl and 140 mM NaCl at pH 7.4. The reaction was incubated for 0, 30, 60, 120, 180, or 240 min at 37 °C. The reactions were quenched with $ZnCl_2$ (10mM final concentration), and the products were analyzed by HPLC on a Vydac C18 column using a 5% to 50% gradient of 0.1% aqueous TFA-acetonitrile. The chromatograms were monitored at 210 nM and 280 nM in order to follow the peptide and vinblastine components, respectively, generated from the conjugates. The difference in the area of the peptide component peak versus the area of the untreated pro-drug peak was used as a measurement of peptide hydrolysis. Results are reported as the time required for PSA to hydrolyze 50% of the initial substrate.

Synthesis of Peptide Precursor 7a to Conjugates 3a−3c, 7b to Conjugate 5, and 7c to Conjugate 6. Each of the three peptide carboxylic acid intermediates **7a**, **7b**, and **7c** was prepared by Merrifield solid-phase peptide synthesis protocols employing double couplings on a Model 430A Applied Biosystems (Foster City, CA) automated peptide synthesizer. The resin for the peptide assembly was 4-hydroxymethylphenoxy (Wang-type HMP) resin, purchased from ABI or Midwest Biotech preloaded with Fmoc-(O-t-Bu)-serine at 0.60−0.85 mmol amino acid per g of resin. N^{α}-Fmoc-protected amino acids of the L-configuration and reagents were supplied by ABI. N^{α}-Fmoc-protected amino acids of the D-configuration, as well as N^{α}-Fmoc-Hyp-OH and N^{α}-Fmoc-Chg-OH, were purchased from Bachem, Inc. Side-chain protection was Ser(t-Bu), D-Ser(t-Bu), Gln(Trt), and D-Gln(Trt). Amino acids were coupled in NMP as solvent, using DCC and HOBt. ABI software version 1.41 for NMP/HOBt Fmoc chemistry was used, starting with 0.5 mmol of resin-bound Fmoc-(O-t-Bu)-serine and employing a 2-fold excess of activated protected amino acid for each coupling and recoupling. Following completion of the assembly on the resin support, the N-terminal Fmoc group was removed via the standard 25% piperidine/NMP protocol, followed by washing 5 times with NMP and introduction of the N-terminal acetyl group by double coupling with acetic acid, 1 mmol (2 equiv) each coupling. Deprotection and removal of peptides from the solid support were carried out by stirring each resin product in TFA with 2.5% each of H_2O and TIPS added, for 2.0 h at 20 °C.[25] Peptides were isolated, after filtration of the spent resin and removal of most of the TFA under reduced pressure, by precipitation from cold diethyl ether, followed by filtering and drying of the solid in vacuo. Purities were assessed by analytical RP-HPLC (see above). Identities were confirmed by amino acid compositional analysis following hydrolysis with 6N HCl and mass spectral analysis.

The three peptide samples were deemed sufficiently pure for use in further synthetic transformations (see below; *, corr. for peptide content):

Ac-Hyp-Ser-Ser-Chg-Gln-Ser-OH (**7a**): 370 mg (80% yield*); anal. HPLC (system A) 7.4 min, 96%; FAB-MS *m*/*z* 702.3 (calcd $[M + H]^+$ 702.5); amino acid compositional analysis 1.08 μmol/mg peptide (theory/found), Ser 3.0/2.98 (corr.), Glu 1.0/1.03 (Gln → Glu), Chg 1.0/1.01, Hyp 1.0/1.03.

Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-OH (7b): 407 mg (76% yield*); anal. HPLC (system A) 6.6 min, 98%; FAB-MS *m*/*z* 789.3 (calcd $[M + H]^+$ 789.5); amino acid compositional analysis 0.94 μmol/mg peptide (theory/found), Ser 4.0/3.41 (corr.), Glu 1.0/0.96 (Gln → Glu), Chg 1.0/1.03, Hyp 1.0/1.01.

Ac-Hyp-Ser-Ser-Chg-D-Gln-D-Ser-Ser-OH (7c) 475 mg (76% yield*); anal. HPLC (95% A → 50% A over 20 min) 12.9 min, 95%; FAB-MS *m*/*z* 789.2 (calcd $[M + H]^+$ 789.5); amino acid compositional analysis (theory/found), Ser 4.0/3.70 (corr.), Glu 1.0/0.93 (Gln → Glu), Chg 1.0/1.06, Hyp 1.0/1.01.

4-O-*Des*-acetyl Vinblastine 1b.[12] To a solution of 2.40 g of vinblastine sulfate salt 1 (Sigma Chemical Co. V-1377) in 135 mL of absolute CH_3OH under N_2 was added 45 mL of anhydrous hydrazine, followed by stirring at 22 °C for 16 h. Analysis by HPLC (system A) showed approximately 95% dAc-VIN (**1b**), RT 24.4 min, and 4% of the product of further hydrazinolysis at the 23-carboxymethyl group.[11] The reaction was evaporated to a thick paste, which was partitioned between 300 mL of CH_2Cl_2 and 150 mL of saturated $NaHCO_3$.

The bicarbonate layer was washed twice with 100 mL portions of CH_2Cl_2, and each of the 3 CH_2Cl_2 layers was washed in turn with 100 mL each of H_2O (2X) and saturated NaCl (1X). The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed at reduced pressure to yield 1.82 g (90% yield) of the title compound as an off-white crystalline solid: retention time (system A) 23.9 min, 95% pure, high-resolution ES/FT-MS *m/e* 769.7. This material was kept stored at −20 °C until further use.

A 260 mg sample dissolved in 60 mL of 5% HOAc was purified and isolated as the acetate salt by preparative HPLC on a Waters C18 Delta-Pak column 15μ 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 70% A/70 min at a flow rate of 80 mL/min. The lyophilizate from pooled homogeneous fractions was passed through *BioRad* AG4 × 4 ion-exchange resin (acetate) to give the title compound as the HOAc salt: retention time (system C) 24.6 min, 99.5% pure, FAB-MS *m/z* 769.4 (calcd $[M + H]^+$ 769.5).

4-O-(Leucyl)-*des*-acetyl Vinblastine 2a. A solution of 261 mg (1.00 mmol) of N-phthaloyl leucine in 3 mL of THF was cooled to −15 °C under N_2 and treated with 0.14 mL of TEA, followed by 0.13 mL of isobutyl chloroformate. After stirring for 30 min, a solution of 100 mg (0.13 mmol) of dAc-VIN **1b** in 5 mL of 1:1 THF/ethyl acetate was added, and the reaction mixture was let warm to 20 °C over 1 h, then stirred for 20 h. The solvent was removed by evacuation under reduced pressure, and 10 mL of methanol was added and removed under reduced pressure, repeating twice more. To the residue dissolved in 30 mL of methanol was added 2 mL of anhydrous hydrazine, and the solution was kept for 20 h at 25 °C. The methanol was removed under reduced pressure, and the crude product was purified by dissolving in HOAc/acetonitrile/ H_2O and passing through preparative HPLC on a Waters C18 Delta-Pak column 15μ 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 50% A/60 min at a flow rate of 80 mL/min to give 60 mg of the title compound as the TFA salt: retention time (system B) 16.6 min, 99% pure, high-resolution ES/FT-MS *m/z* 882.5 (calcd $[M + H]^+$ 882.7).

4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Leu)-*des*-acetyl Vinblastine 3a. A sample of 60 mg (0.050 mmol) of 4-O-(leucyl) *des*- acetyl vinblastine TFA salt **2a** was dissolved in 3 mL of NMP under N_2, along with 80 mg (0.12 mmol) of peptide **7a**, and the solution was cooled to 0 °C. Then 16.2 mg (0.10 mmol) of HOAt was added, followed by NMM to ensure a pH of ≥6.5 (2 drops). After dissolution was complete, 23 mg (0.12 mmol) of EDC was added, followed by stirring of the solution at 0−5 °C. The coupling was shown to be nearly complete after 8 h, as monitored by analytical HPLC (system B). After 20 h the reaction was worked up by addition of 1 mL of H_2O and, after stirring 1 h, concentrated to a small volume in vacuo. Upon dissolving in ca. 40 mL of 5% HOAc, the sample was subjected to preparative HPLC on a Waters C18 Delta-Pak column 15mM 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 50% A/70 min) at a flow rate of 80 mL/min. Homogeneous fractions were pooled, concentrated to a small volume in vacuo, and lyophilized to yield 25 mg of white powder: anal. HPLC (system B) 19.0 min, 99%; ES/FT-MS *m/z* 1565 (calcd $[M + H]^+$ 1565.2); amino acid compositional analysis (theory/found), Ser 3.0/2.90 (corr.), Glu 1.0/1.00 (Gln → Glu), Chg 1.0/1.03, Hyp 1.0/1.05, Leu 1.0/1.00.

4-O-(Glycyl)-*des*-acetyl Vinblastine 2b. A solution of 205 mg (1.00 mmol) of N-phthaloyl glycine in 6 mL of ethyl acetate was cooled to −15 °C under N_2 and treated with 0.14 mL of TEA, followed by 0.13 mL of isobutyl chloroformate. After stirring for 30 min, a solution of 100 mg (0.13 mmol) of dAc-VIN **1b** was added as a solid, and the reaction mixture was let warm to 20 °C over 1 h, then stirred for 24 h. The solvent was removed by evacuation under reduced pressure, and 10 mL of methanol was added and removed under reduced pressure, repeating twice more. To the residue dissolved in 15 mL of methanol was added 0.2 mL of anhydrous hydrazine, and the solution was kept for 20 h at 25 °C. The methanol was removed under reduced pressure, and the crude product was purified by dissolving in HOAc/acetonitrile/ H_2O and passing through preparative HPLC on a Waters C18 Delta-Pak column 15μ 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 50% A/60 min at a flow rate of 80 mL/min to give 80 mg of the title compound as the TFA salt: retention time (system B) 14.6 min, 99% pure, high-resolution ES/FT-MS *m/z* 826.5 (calcd $[M + H]^+$ 826.6).

4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Gly)-*des*-acetyl Vinblastine 3b. A sample of 70 mg (0.066 mmol) of 4-O-(glycyl) *des*-acetyl vinblastine TFA salt **2b** was dissolved in 3 mL of NMP under N_2, along with 106 mg (0.16 mmol) of peptide **7a**, and the solution was cooled to 0 °C. Then 21.4 mg (0.16 mmol) of HOAt was added, followed by NMM to ensure a pH of ≥6.5 (2 drops). After dissolution was complete, 30 mg (0.16 mmol) of EDC was added, followed by stirring of the solution at 0−5 °C until completion of the coupling as monitored by analytical HPLC (system B), approximately 2 h. After 20 h the reaction was worked up by addition of 1 mL of H_2O and, after stirring 1 h, concentrated to a small volume in vacuo. Upon dissolving in ca. 40 mL of 5% HOAc, the sample was subjected to preparative HPLC on a Waters C18 Delta-Pak column 15mM 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 50% A/60 min at a flow rate of 80 mL/min. Homogeneous fractions were pooled, concentrated to a small volume in vacuo, and lyophilized to yield 41 mg of white powder: anal. HPLC (system B) 16.1 min, 99%; ES/FT-MS *m/z* 1508 (calcd $[M + H]^+$ 1507.6); amino acid compositional analysis (theory/found), Ser 3.0/3.06 (corr.), Glu 1.0/1.00 (Gln → Glu), Chg 1.0/1.02, Hyp 1.0/1.05, Gly 1.0/1.05.

4-O-(Prolyl)-*des*-acetyl Vinblastine 2c. A solution of 13.1 g (38.9 mmol) of Fmoc-Pro-OH (Bachem, Inc.) in 100 mL of dry CH_2Cl_2, cooled to 0 °C under N_2, was treated with 6.7 mL (9.74 g, 76.8 mmol) of oxalyl chloride and 0.1 mL of DMF, letting warm to 15−20 °C over a period of 2 h. Solvent removal under reduced pressure, followed by flush/evacuation twice with anhydrous CH_2Cl_2 and drying to constant weight in vacuo, afforded the acid chloride as an off-white solid, which was usable without purification and kept stored at −20 °C until use.

A sample of 804 mg (1.047 mmol) of 4-O-*des*-acetylvinblastine (**1b**), dissolved in 3 mL of CH_2Cl_2 and 18 mL of anhydrous pyridine under nitrogen, was treated with 1.39 g (3.7 mmol) of Fmoc-Pro-Cl (above), and the mixture was stirred for 20 h at 25 °C. The progress of the reaction was determined by disappearance of the starting dAc-VIN upon analytical HPLC (system B). Approximately 3 mL of H_2O was added to react with excess acid chloride, and the solution was then evaporated to a thick orange oil and partitioned between 300 mL of EtOAc and 150 mL of saturated $NaHCO_3$, followed by washing twice with saturated NaCl. After drying (Na_2SO_4), the solvent was removed under reduced pressure to give an orange-brown residue, to which was added 30 mL of DMF and 14 mL of piperidine, and after 5 min the solution was evaporated under reduced pressure to give a orange-yellow solid residue. After this material was dried in vacuo for about 1 h, approximately 200 mL of H_2O and 100 mL of ether were added, followed by glacial HOAc dropwise with shaking and sonication until complete dissolution had occurred and the aqueous layer had attained a pH of 4.5−5.0 (moistened pH range 4−6 paper). The aqueous layer was then washed twice with 100-ml portions of ether, and each ether layer was washed in turn with 50 mL of H_2O. The combined aqueous layers were freed of ether by evacuation of about 20% of the total volume under reduced pressure, and the remaining aqueous solution was subjected to preparative HPLC in 2 portions on a Waters C18 Delta-Pak column 15μ 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 70% A/70 min at a flow rate of 80 mL/min. Pooled fractions yielded, upon concentration and lyophilization, 810 mg (64% yield) of the title compound as the TFA salt: retention time (system A) 23.5 min, 96.2% pure, high-resolution ES/FT-MS *m/z* 866.6 (calcd $[M + H]^+$ 866.).

A 110-mg sample was purified and isolated as the acetate salt by preparative HPLC on a Waters C18 Delta-Pak column 15μ 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN),

gradient elution 95% → 70% A/70 min at a flow rate of 80 mL/min. The lyophilizate from pooled homogeneous fractions was passed through *BioRad* AG4 × 4 ion-exchange resin (acetate) to give the title compound as the HOAc salt: retention time (system C) 23.4 min, 99.8% pure; FAB-MS *m*/*z* 866.5 (calcd $[M + H]^+$ 866.7); amino acid compositional analysis (theory/found) Pro 1.08/1.01 mmol/gm.

4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Pro)-*des*-acetyl Vinblastine 3c. A sample of 30.5 mg (0.025 mmol) of 4-O-(prolyl) *des*-acetyl vinblastine TFA salt **2c** was dissolved in 6 mL of DMF under N_2, along with 40.3 mg (0.06 mmol) of peptide **7a**, and the solution was cooled to 0 °C. Then 13.5 mg (0.10 mmol) of HOAt was added, followed by 2,4,6-collidine to ensure a pH of ≥6.5 (3 drops). After dissolution was complete, 13.2 mg (0.07 mmol) of EDC was added, followed by stirring of the solution at 0−5 °C until completion of the coupling as monitored by analytical HPLC (system A), approximately 8 h. After 20 h the reaction was worked up by addition of 1 mL of H_2O and, after stirring 1 h, concentrated to a small volume in vacuo. Upon dissolving in ca. 40 mL of 5% HOAc, the sample was subjected to preparative HPLC on a Waters C18 Delta-Pak column 15mM 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3-CN), gradient elution 85 → 65% A/70 min at a flow rate of 80 mL/min. Homogeneous fractions were pooled, concentrated to a small volume in vacuo, and lyophilized to yield 25 mg (65% yield) of white powder: anal.HPLC (system A) 27.0 min, 99.3%; ES/FT-MS *m*/*z* 1550.0 (calcd $[M + H]^+$ 1550.2); amino acid compositional analysis (theory/found), Ser 3.0/2.60 (corr.), Glu 1.0/0.97 (Gln → Glu), Chg 1.0/1.02, Hyp 1.0/1.08, Pro 1.0/0.94.

Preparation and Comparison of Stabilities of 4-O-(Ser-Leu)-*des*-acetyl Vinblastine 4a and 4-O-(Ser-Pro)-*des*-acetyl Vinblastine 4c. Samples of 6.3 mg each of 4-O-(Leu)-dAc-VIN 2a and 4-O-(Pro)-dAc-VIN 2c, respectively, were dissolved in 3 mL of DMF, along with 2.8 mg of Boc-Ser-OH, followed by 2.7 mg of HOAt and 1 drop of 2,4,6-collidine, then 2.6 mg of EDC. After 22 h analytical HPLC (system B) showed the reaction to be complete, and 1 mL of H_2O was added. In 1 h the solvent was removed in vacuo, and the intermediate 4-O-(Boc-Ser) derivative was isolated by partitioning with EtOAc/H_2O and washing in turn with dil. $KHSO_4$, H_2O, $NaHCO_3$, and NaCl (2X), followed by drying over Na_2SO_4, solvent removal and drying in vacuo. Treatment with 3 mL of TFA/CH_2Cl_2 (2:1 v/v), then evacuation under reduced pressure and dissolution in 1.0 mL of H_2O, afforded the title compounds: **4a** HPLC (system B) 28.1 min (95.4% pure); **4c** HPLC (system B) 23.3 min (96.7% pure).

The pH of each solution was adjusted to 7.5 by the addition of saturated $NaHCO_3$. After 1 h **4c** had been converted to dAc-VIN **1b** (RT 24.3 min) to the extent of 8.6%, at 4 h 31.8%, and at 24 h nearly completely, corresponding to a $T_{1/2}$ of approximately 6 h. Within the same 24 h period, **4a** had generated ≤0.5% of **1b**.

4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-Pro)-*des*-acetyl Vinblastine 5. Samples of 522 mg (0.66 mmol) of peptide carboxylic acid **7b** and 555 mg (0.46 mmol) of 4-O-(Pro)-dAc-VIN **2c**, prepared as above, were dissolved in 17 mL of DMF under N_2. Then 163 mg (1.13 mmol) of HOAt was added, and the pH was adjusted to 6.5−7 (moistened 5−10 range pH paper) with NMM, followed by cooling to 0 °C and addition of 155 mg (0.81 mmol) of EDC. Stirring was continued at 0−5 °C until completion of the coupling as monitored by analytical HPLC (system A), approximately 8 h, maintaining the pH at 6.5−7 by periodic addition of NMM. Analysis showed the major component at 26.3 min preceded by a significant minor component (ca. 20%) at 26.1 min, suggestive of possible racemization at the C-terminal serine during the coupling. After 12 h the reaction was worked up by addition of ∼4 mL of H_2O and, after stirring 1 h, concentrated to a small volume in vacuo and dissolution in 150 mL of 5% HOAc, followed by preparative HPLC in two portions on a Waters C18 Delta-Pak column 15 μM 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 95% → 65% A/70 min. Homogeneous fractions from both runs were pooled and concentrated to a volume of ∼50 mL and passed through approximately 40 mL of *Biorad* AG4 × 4 ion-exchange resin (acetate cycle), followed by freeze-drying to give 300 mg (38% isolated yield) of the title compound as a lyophilized powder: anal. HPLC (system A) 26.7 min, 98.8% (<1% D-Ser isomer, 26.0 min); ES/FT-MS *m*/*z* 1637.0 (calcd $[M + H]^+$ 1636.8); amino acid compositional analysis (theory/found), Ser 4.0/2.54 (uncorr.), Glu 1.0/1.01 (Gln → Glu), Chg 1.0/1.09, Hyp 1.0/0.88, Pro 1.0/1.03.

Fractions containing at least 90% of the title major component were combined and concentrated in vacuo, then repassed through preparative HPLC as above. Pooled fractions containing <2% D-Ser isomer, after concentration and ion exchange as above, yielded an additional 100 mg (13% yield) of the title compound.

In a scaled-up preparation of conjugate **5**, modifications were implemented to reduce the extent of racemization at the C-terminal serine of peptide **7b**. Thus, when 4.95 g (6.28 mmol) of peptide carboxylic acid **7b** and 4.50 (3.73 mmol) of 4-O-(Pro)-dAc-VIN **2c** were coupled in 300 mL of DMF using 1.72 g (10.5 mmol) of the additive ODHBt, using NMM as base and 1.88 g (9.8 mmol) of EDC, the extent of racemization, determined by monitoring by HPLC as above, was reduced to <10%, as determined by analytical HPLC (system A) of the crude product prior to preparative HPLC.

4-O-(Ac-Hyp-Ser-Ser-Chg-D-Gln-D-Ser-Ser-Pro)-*des*-acetyl Vinblastine 6. Samples of 125 mg (0.16 mmol) of peptide carboxylic acid **7c** and 130 mg (0.13 mmol) of 4-O-(Pro)-dAc-VIN **2c**, prepared as above, were dissolved in 10 mL of DMF under N_2. Then 44 mg (0.27 mmol) of ODHBT was added, and the pH was adjusted to 6.5−7 (moistened 5−10 range pH paper) with NMM, followed by cooling to 0 °C and addition of 49 mg (0.25 mmol) of EDC. Stirring was continued at 0−5 °C until completion of the coupling as monitored by analytical HPLC (system A), approximately 4 h. Analysis showed the major component at 25.9 min. After 24 h the reaction was worked up by concentrating to a small volume in vacuo and dissolving in 50 mL of 5% HOAc, followed by preparative HPLC on a Waters C18 Delta-Pak column 15μM 300A (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN), gradient elution 85 → 65% A/90 min. Homogeneous fractions were pooled and concentrated to a volume of ∼50 mL and passed through approximately 30 mL of *Biorad* AG4 × 4 ion-exchange resin (acetate cycle), followed by freeze-drying to give 77 mg (33% isolated yield) of the title compound as a lyophilized powder: anal.HPLC (system A) 24.6 min, 98.9%; ES/FT-MS *m*/*z* 1636.8 (calcd $[M + H]^+$ 1636.8); amino acid compositional analysis (theory/found), Ser 4.0/3.59 (corr.), Glu 1.0/0.94 (Gln → Glu), Chg 1.0/1.06, Hyp 1.0/1.05, Pro 1.0/0.96.

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