Tetrahedron 65 (2009) 312–317

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Laccase-catalyzed coupling of catharanthine and vindoline: an efficient approach to the bisindole alkaloid anhydrovinblastine

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ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 3 October 2008
Accepted 16 October 2008
Available online 25 October 2008

ABSTRACT

The efficient enzyme-catalyzed coupling of the indolic alkaloids catharanthine and vindoline was carried out by exploiting the oxidoreductases laccases and atmospheric oxygen. Following $NaBH_4$ reduction of the eniminium cationic intermediate, the synthetically useful dimer anhydrovinblastine (AVBL) was isolated and characterized. Several reaction parameters were investigated in detail and, under the optimized reaction conditions, AVBL was isolated in 56% yield. The practicability of this bioconversion was further confirmed through the condensation of catharanthine with the vindoline analogue 11-methoxydihydrotabersonine.

1. Introduction

The leaves of *Catharanthus roseus* contain a large number of bisindole alkaloids, among which vinblastine (VBL, **1**) can be isolated in trace quantities (0.00025% of dry leaf weight) after tedious and accurate chromatographic separations.[1] VBL has been shown to be an important antitumor compound and, also today, is in widespread use for the treatment of various carcinomas.[2] However, the low occurrence of **1** in plants and its complex isolation procedure make it difficult to provide this pharmacological agent in acceptable yields and at low cost. Thus, the synthesis of VBL was, and still remains, an important and fascinating target in organic chemistry.

Total syntheses of **1** have been developed by different groups,[3] but they can be considered more a chemical exercise than a practical approach, because of the excessive number of synthetic steps and of the consequently low isolated yields. Conversely, few research groups have examined its semi-synthesis from more available natural precursors. All the reported approaches to **1** rely on the apparently simple formal addition of water to the $C_{15'}$–$C_{20'}$ double bond of the recognized biosynthetic precursor anhydrovinblastine (AVBL, **2**, Scheme 1, path A).[4] This synthetic goal has been achieved via multi-step sequences, but the formation of **1** still occurs in moderate to low yields and has been inevitably accompanied by side products, amongst which the C-20′ epimer of VBL (leurosidine) and other dimeric alkaloids have been detected.[5–8] In a series of papers, Kutney and co-workers described a 'bio'-approach for the transformation of AVBL into VBL, exploiting bioconversions catalyzed by whole *C. roseus* cell cultures or by their crude enzymes' cocktail (free or immobilized). An innovative synthesis has been reported (16% yield of **1** from **2**), which alternates oxidation and reduction steps, with oxygen and $NaBH_4$, respectively, in the presence of an anti-VBL monoclonal antibody acting as a chiral template.[7] Similarly, a further significant approach to VBL has been very recently proposed by Boger and co-workers.[8] The preparation of **1** from **2** in 48% isolated yield (on a 0.08 mmol scale) was achieved by using a mixture of iron(III) oxalate hexahydrate, air, and $NaBH_4$, but, once again, the reaction mixture also contained leurosidine (18%) and 20′-deoxyleurosidine (6%).

In turn, the recognized central role of AVBL in the formation of VBL, as well as of the semi-synthetic antitumoral alkaloid vinorelbine (**3**, Scheme 1, path B)[9] together with AVBL low natural availability,[10] has spurred many efforts towards its synthesis. The most efficient approach to **2** is based on the emulation of the biosynthetic pathway, and consists in the condensation of the monomeric alkaloids catharanthine (**4**) and vindoline (**5**),[11] both occurring as more frequent constituents in plants. In this biomimetic method, **4** is oxidized to generate an electrophilic species (**6**) that is captured by the nucleophile **5** to give a dimeric cationic intermediate **7** that, after $NaBH_4$ reduction, gives AVBL (Scheme 2). Different strategies have been examined to initiate the reaction sequence of Scheme 2, exploiting electrochemical,[12] photochemical,[13] chemical, or enzymatic approaches.

Chemical oxidation of **4** can be achieved by peracids (the so-called Potier–Polonovski route[14]) or Fe^{3+} salts.[15] Whereas in the

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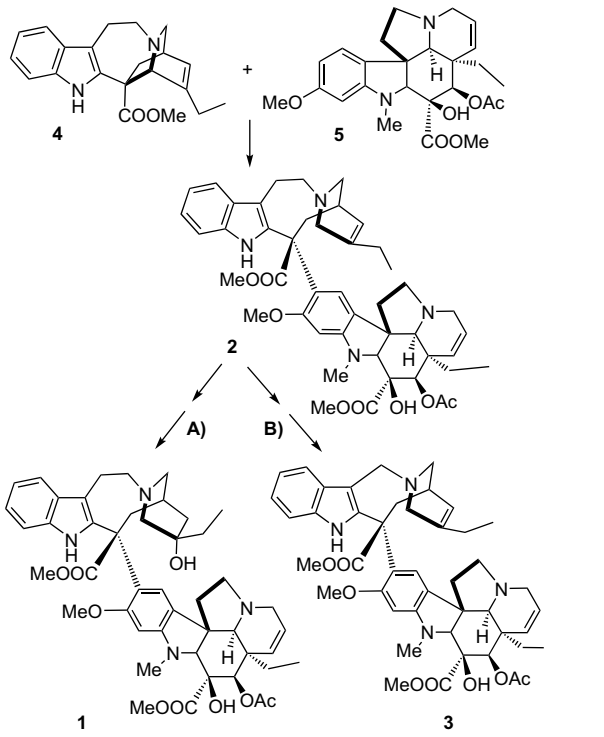
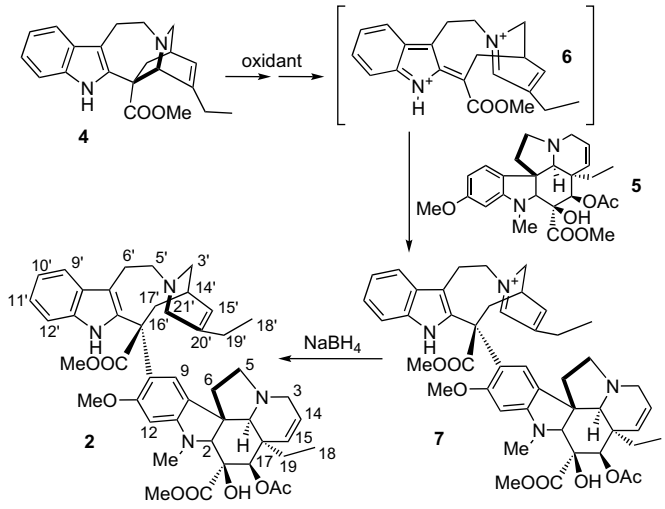
doi:10.1016/j.tet.2008.10.064



Scheme 1. Synthesis of vinblastine (**1**) and vinorelbine (**3**) from catharanthine (**4**) and vindoline (**5**) via anhydrovinblastine (**2**).

Potier–Polonovski process the stereoselectivity is temperature dependent (at low *T*, −78 °C, the 16′-(*S*) isomer is formed, while at 0 °C the 16′-(*R*) epimer predominates), the coupling reaction is stereoselective for the formation of the desired 16′-(*S*) isomer when using $FeCl_3$ as an oxidant, and, besides that, yields are significantly higher.[8]

Crude enzymes obtained from suspension cultures of *C. roseus* catalyzed the coupling of **4** and **5** to give AVBL in ca. 25% yields.[16] The enzymes involved in the process were partially purified and their peroxidase nature was demonstrated;[17] therefore it is not surprising that the next step was the investigation of the performances of a series of commercially available peroxidases or heme-containing oxidases.[18] Interesting results were obtained with horseradish peroxidase, microperoxidase, and hemin: under optimized conditions a 58% conversion was estimated by HPLC.



Scheme 2. Synthetic sequence to produce anhydrovinblastine (**2**) from catharanthine (**4**) and vindoline (**5**).

However, the isolated yields were not reported in these papers, and, additionally, the authors recognized the need for a precise control of the reaction conditions, and that could represent a problem for the scaling up of the biotransformation.

Laccases are an interesting group of copper-containing oxidoreductases (the so-called 'blue oxidases') widely distributed in nature. They are highly stable and catalyze the oxidation of a wide range of substrates via the use of atmospheric oxygen and under very mild reaction conditions.[19] Most of the reported applications of these enzymes are related to the oxidation of phenolic derivatives to give dimeric products.[20] Of synthetic relevance are also a series of papers describing the nuclear amination, domino, or Diels–Alder reaction of laccase-generated quinones,[21] thus showing that it is possible to exploit these enzymes to produce reactive intermediates in situ. There are also some scant data on the oxidation of amines,[22] and, as far as alkaloids are concerned, in the 1980s Rosazza described the oxidation of vindoline catalyzed by a laccase from *Polyporus anceps* to give the oxygen-bridged derivative **5a** and the bisindole **5b** (Fig. 1).[23]

Based on our experience in indole alkaloid chemistry[24] and in the use of enzymes in organic synthesis,[25] and specifically of laccases,[20] we thought that these latter biocatalysts could replace chemical reagents in the oxidative activation of catharanthine, thus offering a new mild strategy to AVBL production. This enzymatic approach was mentioned in a review on laccases published in the early 1990s,[26] but, to the best of our knowledge, it was never explored in practice. Here, we show that indeed laccases can efficiently catalyze the preparative-scale condensation of **4** with **5**, and we have extended this approach to the synthesis of an AVBL analogue.

2. Results and discussion

Preliminary experiments were performed to collect information on substrate reactivity in the presence of a laccase from *Trametes pubescens* (*Tp*). Accordingly, both **4** and **5** were separately incubated with the enzyme following previously described protocols,[20] and the reactions were monitored by TLC and RP-TLC (for details see Supplementary data). We found that both alkaloids were accepted by the laccase: whereas catharanthine gave origin to numerous unidentified byproducts, vindoline was transformed into two derivatives that could be isolated and identified as the expected and previously described compounds **5a** and **5b**.[23]

In a subsequent experiment, catharanthine (0.13 mmol) and vindoline (0.11 mmol) were dissolved in 20 mM acetate buffer pH 4.5 (10 mL). A sample of *Tp* laccase (300 U) was added and the solution was gently stirred at 30 °C for 8 h. The reaction was then quenched with $NaBH_4$, the solution was made mildly alkaline, and extracted with CH_2Cl_2. The crude residue was purified by silica gel chromatography and the main product (0.025 mmol) was identified as the expected anhydrovinblastine (**2**). Diagnostic structural data were the HRESI mass at 793.41492 Da (theoretically for $C_{46}H_{57}N_4O_8$,

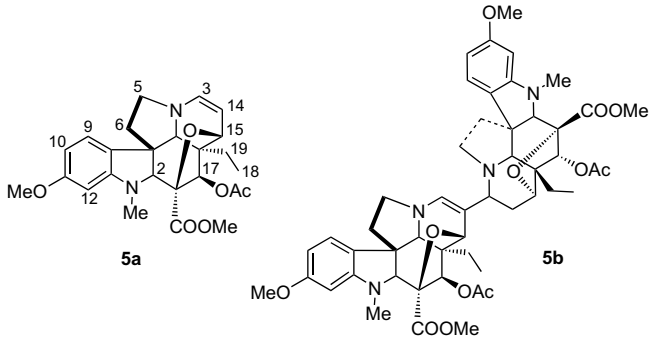


Figure 1. Compounds **5a** and **5b**.

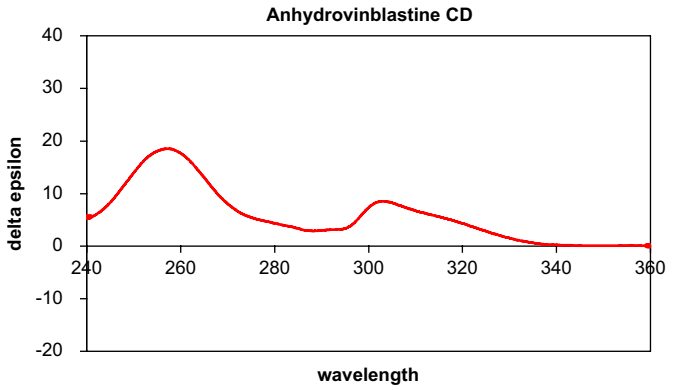


Figure 2. Circular dichroism spectrum of AVBL (**2**). λ_{max} 258 nm, $\Delta\varepsilon$=+18.4; λ_{max} 305 nm, $\Delta\varepsilon$=+8.2.

$[M+H]^+$, 793.41709) and, in addition to the expected ^1H and ^{13}C NMR aliphatic and aromatic signals (see Section 3), the correct configuration at C-16′ was assigned by circular dichroism (CD) analysis. In the 1970s, Potier pointed out that CD allows a ready distinction to be made between the 16′-(*S*) and 16′-(*R*) epimers, with the delta epsilon maximum at 258 nm being positive ($\Delta\varepsilon$ +14.0) or negative ($\Delta\varepsilon$ −13.0), respectively.[14a] Our isolated product showed a positive maximum at 258 nm ($\Delta\varepsilon$ +18.4), thus clearly confirming the presence of the correct 16′-(*S*) stereocenter (Fig. 2).

Being pleased with this positive result, we focused our efforts on the optimization of the reaction conditions. Initially, we tested a series of commercially available laccases (from *Trametes versicolor*, *Tv*; *Rhus vernicifera*, *Rv*; *Pyricularia oryzae*, *Po*; *Agaricus bisporus*, *Ab*; *Myceliophtora termophila*, *Mt*) and, under the same reaction conditions, we found that the laccases from *Trametes* (both *Tv* and the previously tested *Tp*) were by far the best biocatalysts and actually the only ones that produced a significant amount of the eniminium **7**, precursor of AVBL. Minor production of **7** was observed with *Po*, *Ab*, and *Mt* laccases (5–12% of the amount produced by *Tv* or *Tp*), whereas this compound was not detected in the presence of *Rv* laccase. As *Tp* and *Tv* laccases performed very similarly, the subsequent experiments were conducted in the presence of the commercial enzyme isolated from *T. versicolor*. Several other parameters were investigated in detail (buffer pH and ionic strength, temperature, stirring or shaking, oxygenation). It was found that best results were obtained using a 50 mM acetate buffer at pH 4.0, maintaining the reaction under mild shaking at 30 °C in the presence of atmospheric air. A detailed investigation on the influence of different water-miscible organic solvents on laccase stability and activity was also undertaken, but no positive effects on this specific coupling reaction were observed in the presence of any of the solvents tested (acetone, acetonitrile, dioxane, DMF, DMSO, EtOH, MeOH, *i*-PrOH, THF).

Under the optimized reaction conditions, the laccase-mediated condensation of **4** and **5** was repeated on a preparative scale using 440 mg of each substrate (0.96 mmol of **5** and 1.31 mmol of **4**), and, following the usual work-up and chromatography, AVBL could be isolated in 56% yield (415 mg, 0.54 mmol). The HPLC chromatograms of the reaction mixture at various stage of the process are shown in Figure 3.

Subsequently, in order to be able to selectively remove the oxidative catalyst out of the reaction solution and to recycle the enzyme, *Tv* laccase was immobilized on an Eupergit© carrier. The immobilized enzyme behaved similar to the free laccase, it could be separated by simple filtration and reused for five reaction cycles without significant activity loss.

Finally, the scope of this laccase-catalyzed bioconversion was expanded by studying the condensation of catharanthine with the vindoline analogue 11-methoxy-dihydrotabersonine (**8**, Scheme 3). The laccase-catalyzed reaction worked smoothly: the expected heterodimeric alkaloid **9** was isolated in 21% yield and its structure confirmed by NMR and mass analyses.[27]

In conclusion, we have shown that commercially available laccases can catalyze on a preparative scale the efficient coupling of indole alkaloids using inexpensive air oxygen under very simple experimental protocols. AVBL isolated yield was higher than in previously proposed enzymatic protocols and compared well with the reported chemical, electrochemical, and photochemical approaches.

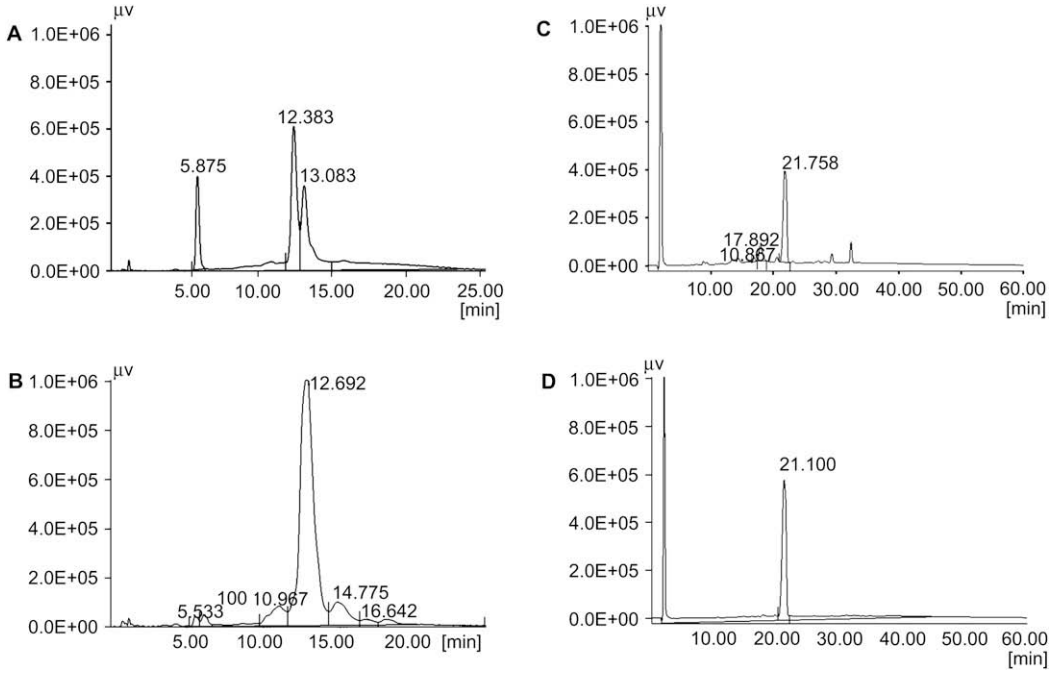
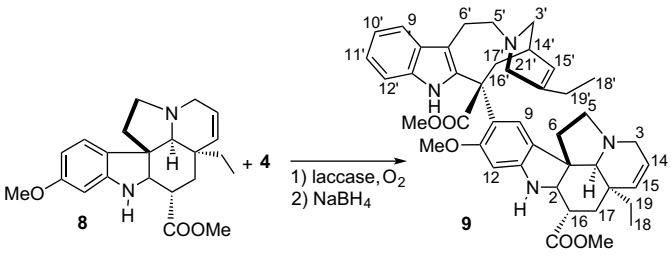


Figure 3. Laccase-catalyzed condensation of catharanthine (**4**) and vindoline (**5**), HPLC analysis of the reaction progress: (A) after 1.5 h, (B) after 8 h (reaction end), (C) following reduction with $NaBH_4$, and (D) purified **2**.



Scheme 3. Laccase-mediated coupling of 11-methoxy-tabersonine (**8**) and catharanthine (**4**).

3. Experimental section

3.1. Material and methods

Catharanthine (**4**) and vindoline (**5**) were from Chemateck S.p.A (Milano, Italy), while 11-methoxy-dihydrotabersonine (**8**) was a kind gift of Dr. Bogomil Pyuskyulev (Academy of Sciences, Sofia, Bulgaria). Laccase from *T. pubescens* was provided by Prof. Haltrich (BOKU University, Wien, Austria); laccases from *T. versicolor*, *R. vernicifera*, *P. oryzae*, and *A. bisporus* were from Sigma–Aldrich, whereas the enzyme from *M. termophila* was from Novozymes. The other substrates and reagents were from Sigma–Aldrich. Eupergit C250L was a gift from Rohm GmbH (Darmstadt, Germany). Enzymatic activities were monitored using a Jasco V-530 UV/VIS spectrophotometer.

Thin-layer chromatography (TLC) was performed on silica plates (Merck 60 F_{254}). Substrates and products were visualized at 254 nm and/or by plates treatment with the CAS reagent ($(NH_4)_2$-$Ce(SO_4)_3\cdot 2H_2O$, 1 g; 85% $H_3PO_4Ce(SO_4)_2$, 100 mL). Purifications were performed by flash chromatography on silica gel (Merck 60, 230–400 mesh).

HPLC analyses were carried out using a C-18 RP column (LIChroCART® 125-4, Aluspher® 100, 5 μm) and a precolumn (LIChroCART® 4-4, Aluspher® 100, 5 μm). Eluent A: 5 mM phosphate buffer pH 6.5; eluent B: acetonitrile. Elution gradient: from eluent A–eluent B 80:20 to eluent A–eluent B 20:80 in 20 min; same eluent (eluent A–eluent B 20:80) for 20 min; flow rate 1 mL/min; UV detection at 294 nm. Retention time: **4**, 12.38 min; **5**, 5.87 min; **7**, 13.08 min; **2**, 15.22. Alternatively it could be used a C-18 RP column (ZORBAX Eclipse XDB-C18, 4.6×150 mm, 5 μm, Agilent Technologies) and a precolumn (Eclipse XDB-C18, 4.6×12.5 mm, 5 μm, Agilent Technologies). Eluent A: 10% (95% acetonitrile and 5% THF) plus 90% 10 mM ammonium acetate buffer pH 6.5; eluent B: 80% (95% acetonitrile and 5% THF) plus 20% 10 mM ammonium acetate buffer pH 6.5. Elution gradient: from eluent A–eluent B 75:25 to eluent A–eluent B 0:100 in 30 min; same eluent (eluent A–eluent B 0:100) for 5 min; flow rate 1 mL/min; UV detection at 294 nm. Retention time: **4**, 16.17 min; **5**, 15.02 min; **7**, 11.05 min; **2**, 21.10 min.

NMR spectra were recorded on a 400 MHz instruments with Me_4Si as internal standard. High resolution electrospray mass spectra (HRESI-MS) were acquired with an FT-ICR (Fourier Transfer Ion Cyclotron Resonance) instrument equipped with a 4.7 Tesla cryo-magnet. Samples were dissolved in CH_3CN and injected into the instrument equipped with its own ESI source. Spectra were recorded in the HR mode with resolutions ranging from 20,000 to 30,000.

3.2. Spectrophotometric assay of laccase activity

Laccase activity was evaluated by monitoring the oxidation of ABTS at 436 nm, following a standard protocol. An enzymatic solution (10 μL) was added to a 1 mL cuvette containing 20 mM acetate buffer pH 3.5 (890 μL) and ABTS (100 μL of a 10 mM solution of ABTS in H_2O). One enzyme unit is defined as the amount of laccase that oxidizes 1 μmol of ABTS under this condition (λ_{ABTS}=29.3 $mM^{-1}\,cm^{-1}$).

3.3. Immobilization of *T. pubescens* laccase on Eupergit C250L

A sample of the crude enzyme preparation (50 mg, 5.850 total U, specific activity 532 U/mg) was dissolved in 600 μL of 0.1 M phosphate buffer, pH 7.0. Additional phosphate buffer (3.1 mL, 1.17 M) was added to this solution, in order to get a resulting 1 M phosphate buffer, pH 7. A sample of this mother solution (50 μL) was stored below 0 °C to be used as a control.

The polymeric matrix carrying reactive epoxidic groups Eupergit C250L (750 mg) was added to 3.7 mL of this solution and the resulting slurry was stored overnight in the fridge, being mixed at regular interval. After 24 h, the slurry was centrifuged (3.000 rpm, 5 min) and washed three times with 5 mL of 0.1 M phosphate buffer pH 7. The residual laccase activity due to the unbound enzyme was measured by spectrophotometric assay and resulted to be 131 U tot (2.2%). The immobilized enzyme was then re-suspended in a 5 mL solution of 0.3 M ethanolamine in phosphate buffer (1.2 M, pH 7.0) and stored in the fridge for 5 h, being mixed at regular intervals. The solution was centrifuged (3000 rpm, 5 min) and washed three times with 5 mL of 0.1 M phosphate buffer pH 7.0. The immobilized enzyme was stored at 4 °C in a 10 mL acetate buffer (50 mM, pH 4.0).

3.4. Laccase stability in the presence of organic co-solvents

The evaluation of the effect of a fixed concentration (10%, 20%, or 30% v/v) of several water-miscible organic co-solvents (acetone, dioxane, acetonitrile, THF, DMF, DMSO, EtOH, MeOH, *i*-PrOH) on laccase stability was performed by spectrophotometric analysis using the previously described assay conditions. The enzyme (approximately 1 U) was dissolved in 1 mL acetate buffer 20 mM, pH 4.5 in the presence of different amounts of one of the co-solvents, and the solutions were gently shaken at rt for 30 days. Every 24 h samples (10 μL) were taken and the residual activities evaluated and compared to the data obtained in the absence of co-solvents.

3.5. Laccase activity in the presence of organic co-solvents

The evaluation of the effect of a fixed concentration (10%, 20%, or 30% v/v) of several water-miscible organic co-solvents (acetone, dioxane, acetonitrile, THF, DMF, DMSO, EtOH, MeOH, *i*-PrOH) on laccase activity was performed by spectrophotometric analysis using the previously described assay conditions. The total volume of solution in the cuvette was 1 mL, made of 20 mM acetate buffer pH 3.5 (700–900 μL) and the respective co-solvent (100–300 μL). The residual activities were evaluated and compared to the data obtained in the absence of co-solvents.

3.6. Laccase-catalyzed coupling of catharanthine (4) and vindoline (5): optimization of the reaction conditions

The same amounts of reagents and enzymes (catharanthine, 20 mg, 14 mM; vindoline, 20 mg, 11 mM; laccase 40 U) were dissolved in acetate buffer (total volume, 4 mL) and the following reaction parameters were changed: buffer ionic strength (20, 50, 100 mM), buffer pH (3.0, 3.5, 4.0, 4.5), temperature (20, 30, 40 °C), shaking (0, 50, 120, 200 rpm), enzyme source (laccase from *T. pubescens*, *T. versicolor*, *M. termophila*, *R. vernicifera*, *P. oryzae*, *A. bisporus*), oxygenation (water monophase (no air phase), static air–water biphasic system (1:1 v/v), air continuously bubbled into the reaction vessel), and organic co-solvent (20% v/v: acetone, MeOH,

i-PrOH, DMSO, dioxane). The best results were obtained using a *Trametes* laccase in 50 mM acetate buffer, pH 4.0, mildly shaken (120 rpm) at 30 °C under oxygenating conditions (1:1 v/v air–water biphasic system or continuous aeration) and without organic co-solvents.

3.7. Oxidation of vindoline (5) catalyzed by *T. versicolor* laccase

Vindoline (**5**, 50 mg, 0.11 mmol) was dissolved in 20 mM acetate buffer, pH 4.5 (10 mL). *T. versicolor* laccase (22 U) was added and the reaction mixture was mildly shaken at 30 °C for 10 h. The formation of two products was detected by TLC (eluent AcOEt–MeOH, 85: 15, R_f (**5**)=0.45, R_f (**5a**)=0.64, R_f (**5b**)=0.73). The reaction mixture was extracted with CH_2Cl_2, the solvent evaporated, and the crude residue purified by silica gel chromatography (eluent hexane–AcOEt–MeOH 8:2:1) to give **5a** and **5b** (5 mg each). The products were analyzed by NMR and their structures confirmed by comparison with the literature data.[22]

Compound ***5a***. ^{1}H NMR (acetone-d_6) selected data δ: 7.12 (1H, d, J=8.0 Hz, H-9), 6.24 (1H, d, J=8.0 Hz, H-10), 6.11 (1H, d, J=7.7 Hz, H-3), 6.04 (1H, s, H-12), 5.30 (1H, s, H-17), 4.86 (1H, dd, J_1=6.5 Hz, J_2=5.8 Hz, H-14), 4.10 (1H, d, J=5.8 Hz, H-15), 2.73 (3H, s, NMe), 1.97 (3H, s, COMe), 0.85 (3H, t, J=7.6 Hz, H-18).

Compound ***5b***. ^{1}H NMR (acetone-d_6) selected data δ: 7.14 (1H, d, J=8.0 Hz, H-9), 7.04 (1H, d, J=8.0 Hz, H-9′), 6.23 (2H, 2d, J=7 Hz, H-10 and H-10′), 6.06 (2H, 2d, J=11 Hz, H-12 and H-12′), 5.38 (2H, d, J=11 Hz, H-17 and H-17′), 2.80 (3H, s, NMe), 2.72 (3H, s, NMe′), 2.02 (3H, s, COMe), 1.93 (3H, s, COMe′), 0.83 (3H, t, J=7.5 Hz, H-18), 0.81 (3H, t, J=7.5 Hz, H-18′).

3.8. Preparative-scale coupling of catharanthine (4) and vindoline (5) catalyzed by *T. versicolor* laccase

Catharanthine (**4**, 440 mg, 1.31 mmol, 1.36 equiv) was suspended in 50 mM acetate buffer (pH 3.5, 41 mL) and vigorously shaken for 10 min at 45 °C in order to dissolve it completely. Vindoline (**5**, 440 mg, 0.96 mmol, 1 equiv) was then added and the reaction solution was shaken for additional 10 min (vindoline was not completely dissolved). The solution pH was adjusted to 4.0 by adding 1 M AcOH. *T. versicolor* laccase (170 mg, 4250 U) was dissolved in 50 mM acetate buffer (pH 4.0, 9 mL) and the solution was dropped into the reaction mixture, which was gently shaken (120 rpm) at 30 °C (vindoline was completely dissolved after 1 h) and monitored by TLC and HPLC.

After 8 h, HPLC analysis revealed the presence of 4% residual vindoline. The solution was filtered to separate the denaturated protein and the pH was adjusted to 7.0 by adding 1 M NaOH. $NaBH_4$ was added (41 mg) and the resulting solution extracted with AcOEt (3×50 mL). The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel chromatography (eluent: *tert*-butyl methyl ether–methanol 85:15 to elute the residual starting materials; *tert*-butyl methyl ether–methanol–TEA 85:15:0.5 to elute anhydrovinblastine) to give 415 mg of anhydrovinblastine (56% isolated yield).

Compound ***2***. ^{1}H NMR (acetone-d_6) δ: 7.48 (1H, d, J=7.9 Hz, H-9′), 7.40 (1H, dd, J=8.0 Hz, H-12′), 7.09 (1H, dd, J_1=7.4 Hz, J_2=7.3 Hz, H-11′), 7.02 (1H, dd, J_1=7.5 Hz, J_2=7.3 Hz, H-10′), 6.84 (1H, s, H-9), 6.39 (1H, s, H-12), 5.81 (1H, br dd, J_1=10.2 Hz, J_2=3.9 Hz, H-14), 5.43 (1H, d, J=6.3 Hz, H-15′), 5.33 (1H, s, H-17), 5.32 (1H, br d, J=9.9 Hz, H-15), 3.85 (3H, s, OMe), 3.71 (3H, s, COOMe (vind)), 3.62 (1H, s, H-2), 3.52 (3H, s, COOMe (cat)), 3.51 (1H, br d, J=14.9 Hz, H-21′a), 3.43 (1H, br d, J=13.3 Hz, H-3′a), 3.25–3.35 (3H, m, H-3a, H-6′a, H-21′b), 3.05–3.25 (5H, m, H-5a, 2H-5′, H-6′b, H-17′a), 2.82 (1H, br s, H-21), 2.73 (3H, s, NMe), 2.70 (1H, br d, J=15.0 Hz, H-3b), 2.55 (2H, br dd, J_1=13.3 Hz, J_2=2.0 Hz, H-3′b), 2.40 (2H, m, H-5b, H-17′b), 2.17 (1H, m, H-6a), 2.00 (3H, s, $OCOCH_3$), 1.95 (2H, m, H-19′), 1.88 (1H, m, H-6b), 1.63 (1H, dq, J_1=14.2 Hz, J_2=7.1 Hz, H-19a), 1.38 (1H, dq, J_1=14.0 Hz, J_2=7.0 Hz, H-19b), 1.02 (3H, t, J=7.3 Hz, H-18′), 0.76 (3H, br t, J=7.0 Hz, H-18). ^{13}C NMR (acetone-d_6) δ: 8.5 (C-18), 12.6 (C-18′), 20.7 (CH_3–COO), 26.1 (C-6′), 28.5 (C-19′), 31.4 (C-19), 34.1 (C-14′), 35.6 (C-17′), 38.4 (N–CH_3), 43.2 (C-20), 45.5 (C-6), 50.9 (C-3), 47.0 (C-3′), 51.0 (C-5), 51.6 and 51.9 (2$COOCH_3$), 53.8 (C-21′), 53.2 (C-7), 54.8 (C-5′), 55.2 (C-16′), 56.2 (OCH_3), 66.1 (C-21), 76.9 (C-17), 80.2 (C-16), 83.9 (C-2), 94.8 (C-12), 111.7 (C-12′), 116.2 (C-7′), 118.6 (C-9′), 119.2 (C-11′), 122.0 (C-10), 121.7 (C-8), 122.3 (C-10′), 124.9 (C-9), 124.7 and 124.8 (C-14 and C-15′), 130.0 (C-8′), 131.7 (C-2′), 130.5 (C-15), 136.2 (C-13′), 140.9 (C-20′), 153.7 (C-13), 158.9 (C-11), 170.4 and 174.9 (2$COOCH_3$), 171.5 ($OCOCH_3$). ESI-MS, positive mode: 793.41492 $[M+H]^+$ (theoretically for $C_{46}H_{57}N_4O_8$ 793.41709). CD: λ_{max} 258 nm, $\Delta\varepsilon$=+18.4; λ_{max} 305 nm, $\Delta\varepsilon$=+8.2 (lit.[13a] λ_{max} 258 nm, $\Delta\varepsilon$=+14.0; λ_{max} 305 nm, $\Delta\varepsilon$=+6.5).

3.9. Coupling of catharanthine (4) and 11-methoxy-dihydrotabersonine (8) catalyzed by *T. versicolor* laccase

Catharanthine (**4**, 30 mg, 0.08 mmol) was dissolved in 20 mM acetate buffer, pH 4.5 (40 mL), while 11-methoxy-tabersonine (**8**, 15 mg, 0.04 mmol) was dissolved in acetone (400 μL). *T. pubescens* laccase (20 U) was dissolved in the same acetate buffer containing **4**, the acetone solution of **8** was added, and the reaction mixture was mildly shaken at 30 °C for 24 h, the conversion was monitored by TLC (eluent AcOEt–MeOH, 85:15, R_f (**4**)=0.54, R_f (**8**)=0.82). $NaBH_4$ (5 mg) was added, the final reaction mixture was extracted with CH_2Cl_2, the solvent evaporated, and the crude residue purified by silica gel chromatography (eluent AcOEt–MeOH 100:1) to give **9** (6 mg) as an amorphous solid. The product was analyzed by NMR and mass spectrometry, and its structure confirmed by comparison with the literature data.[27]

Compound ***9***. R_f (TLC eluent AcOEt–MeOH 85:15) 0.58. ^{1}H NMR (acetone-d_6) δ: 8.60–8.81 (2H, br s, 2 NH), 7.48 (1H, d, J=7.6 Hz, H-9′), 7.30 (1H, d, J=7.6 Hz, H-12′), 7.09 (1H, t, J=7.6 Hz, H-10′), 7.01 (1H, t, J=7.6 Hz, H-11′), 6.69 (1H, s, H-9), 6.32 (1H, s, H-12), 5.78 (1H, dd, J_1=9.0 Hz, J_2=4.5 Hz, H-14), 5.43 (1H, d, J=7 Hz, H-15′), 5.39 (1H, d, J=9.0 Hz, H-15), 4.10 (1H, m, H-2), 3.76 (4H, m, COOMe and H-16), 3.69 (3H, s, OMe), 3.53 (3H, s, COOMe), 2.99–3.52 (10H, m, H-21′a, H-5′b, H-17′a, H-5b, H-6′, H-3′b, H-5′a, H-3b, H-21′b), 2.75–2.89 (1H, m, H-17′b), 2.32–2.63 (3H, m, H-3a, H-3′a, H-5a), 2.26 (1H, br s, H-21), 2.08–2.21 (1H, m), 1.54–2.05 (6H, m, H-17a, H-17b, H-14′, H-19′, H-6a), 1.20–1.31 (1H, m, H-19b), 0.98–1.17 (1H, m H-19a), 1.10 (3H, t, J=7.5 Hz, H-18′), 0.86 (3H, t, J=7.5 Hz, H-18). ^{13}C NMR (acetone-d_6) δ: 141.2 (C-20′), 175.4 (2COOMe), 158.4 (C-11), 152.6 (C-13), 136.0 (C-13′), 135.0 (C-15), 132.8 (C-2′), 130.1 (C-8′), 127.1 (C-8), 125.6 (C-14), 125.2 (C-15′, C-9), 122.6 (C-10′), 120.4 (C-10), 119.6 (C-11′), 118.9 (C-9′), 117.2 (C-7′), 111.5 (C-12′), 94.0 (C-12), 68.4 (C-21), 67.9 (C-2), 56.1 (OMe), 55.1 (C-5′), 54.0 (C-16′), 53.8 (C-21′), 52.5 (C-7), 52.1 (OMe), 51.6 (OMe), 51.3 (C-3, C-5), 47.3 (C-3′), 44.4 (C-6), 43.6 (C-20), 40.8 (C-16), 35.9 (C-17′), 35.0 (C-19), 34.5 (C-14′), 32.5 (C-17), 28.3 (C-6′), 27.8 (C-19′), 12.8 (C-18′), 9.1 (C-18). MS: m/z=705 (M+1).

Acknowledgements

The contribution to this work by Dr. Eleonora Baldelli (INDENA) and Dr. Lara Baratto (ICRM-CNR) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.10.064.

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