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Laccase-mediated oxidation of natural glycosides

Lara Baratto [a], Andrea Candido [a], Mattia Marzorati [a,b], Francesca Sagui [a], Sergio Riva [a,*], Bruno Danieli [b]

[a] *Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milano, Italy*
[b] *Dipartimento di Chimica Organica ed Industriale, Università degli Studi di Milano, Via Venezian 21, 20133 Milano, Italy*

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Abstract

Regioselective oxidations of the primary OH's of natural glycosides (thiocolchicoside, colchicoside, amygdalin, asiaticoside, ginsenoside R_E) have been performed on a preparative scale by exploiting the laccase–2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) methodology. The influence of water-miscible organic cosolvents on the stability and activity of a laccase from *Trametes pubescens* has been investigated. The enzyme has been covalently linked to Eupergit C250L and its performances evaluated. The recovered immobilized enzyme catalyzed several oxidative cycles of thiocolchicoside, without showing significant loss of activity.

Keywords: Laccase; TEMPO; Oxidation; Natural glycosides; Enzyme

1. Introduction

Laccases are oxidoreductases that oxidize a wide range of organic compounds at the expense of molecular oxygen [1]. Typical substrates of these enzymes are amines and phenols, which – following their preliminary oxidation to reactive radical intermediates – undergo chemical coupling to give dimeric and oligomeric derivatives. For instance, we have recently reported on the oxidation of tetrahydro-2-naphthol [2], of the steroidic hormone β-estradiol [3] and of the phytoalexin resveratrol [4].

Laccase oxidation of primary alcohols is also possible, but it is necessary to make use of ancillary chemical "mediators", i.e., 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), violuric acid (VLA) and *N*-hydroxyphthalimide (HPI) [5]: these compounds (used in catalytic amounts) are initially oxidized by the laccase and then the mediator-catalyzed oxidation of primary alcohols takes place [6]. We have recently exploited this methodology for the regioselective oxidation of the primary OHs of sugar derivatives [7]. The efficiency of the system has been initially tested with a series of mono- and disaccharides, and the corresponding glycopyranosiduronates have been isolated and characterized. Subsequently, this chemo-enzymatic approach has been exploited to achieve the partial oxidation of a water-soluble cellulose sample.

Here we report on the extension of this methodology to the regioselective oxidation of natural glycosides. Specifically, reaction optimization (effect of chemical mediators and of organic cosolvents on laccase activity and stability, enzyme immobilization, ...) has been initially investigated with the model compound thiocolchicoside (**1**), and the best conditions have then been used for the selective modification of other target substrates.

2. Experimental

2.1. Material and methods

Thiocolchicoside (**1**), colchicoside (**2**), asiaticoside (**4**) and ginsenoside R_E (**5**) were a gift from Indena S.p.A., Milano, Italy. Amygdalin (**3**) and the other substrates and reagents were from Fluka. Eupergit C250L was a gift from Rohm GmbH (Darmstadt, Germany). Laccase from *Trametes pubescens* was provided by Prof. Haltrich (BOKU University, Wien, Austria) [8]. 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 visual-

* Corresponding author. Tel.: +39 02 2850 0032; fax: +39 02 2890 1239.
E-mail address: Sergio.riva@icrm.cnr.it (S. Riva).

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ized at 254 nm and/or by plates treatment with the molybdate reagent ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 concentrated, 62 mL; made up to 1 L with deionized water). Purifications were performed by flash chromatography on silica gel (Merck 60, 230–400 mesh). HPLC analyses were carried out using a Jasco 880-PU pump equipped with a Jasco 870-UV detector and a Hewlett-Packard HP-3395 integrator. HPLC–MS analyses were carried out using a Bruker Esquire 3000 PLUS (Esi Ion Trap LC/MSn System) connected to an Agilent 1100 HPLC instrument, equipped with a C-18 column pre-capped 3 μm–55 mm; precolumn C-18 Purospher star RP-18e 5 μm. NMR spectra were recorded on a Bruker AC300 (300 MHz) or on a Bruker AC400 (400 MHz). Mass spectra were recorded with a Fourier Transfer Ion Cyclotron Resonance (FT-ICR) APEX^{TM} II model (Bruker Daltonics) equipped with a 4.7 T crio-magnet (Magnex).

2.2. Spectrophotometric assay of T. pubescens 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 ($\varepsilon_{ABTS} = 29.3\ mM^{-1}\ cm^{-1}$).

2.3. Laccase stability in the presence of organic cosolvents

The evaluation of the effect of a fixed concentration (10, 20 or 30%, v/v) of several water-miscible organic cosolvents (acetone, dioxane, acetonitrile, THF, DMF and DMSO) 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 cosolvents, 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 cosolvents.

2.4. Immobilization of T. pubescens laccase on Eupergit C250L

A sample of the crude enzyme preparation (50 mg, 5.850 total U, specific activity 117 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 (3000 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 total U (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 centrifugated (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).

2.5. Oxidation of thiocolchicoside (*1*) with the immobilized laccase

Thiocolchicoside (**1**, 45 mg) was dissolved in 3 mL acetate buffer, 20 mM and pH 4.5. Immobilized laccase (300 μL of the stored suspension) and TEMPO (2.5 mg) were added and the reaction was gently shaken at RT for 24 h. Samples were taken at scheduled times and the oxidation of **1** to **1a** and **1b** was evaluated by HPLC analysis (eluent H_2O (containing 0.05%, v/v, TFA)–acetonitrile 87:13). After 24 h, the immobilized enzyme was recovered by centrifugation, washed extensively with 20 mM acetate buffer, pH 4.5, and reused in a new conversion cycle by addition of fresh substrate solution. Following this protocol, the immobilized biocatalyst was reused for 10 consecutive cycles (24 h each) and the results are summarized in Fig. 3.

2.6. Preparative-scale oxidation of thiocolchicoside (*1*) with T. pubescens laccase

Thiocolchicoside (**1**, 154 mg, 0.27 mmol) was dissolved in 10 mL acetate buffer, 20 mM and pH 4.5. TEMPO (11.4 mg, 0.07 mmol) and 1 mL of enzymatic solution (24 U) were added and the reaction was gently shaken at RT for 8 days, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 8:4:1). The solvent was evaporated and the residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 8:3:0.5 and then 8:4:1) to give 121 mg (0.21 mmol, 76.6% yield, the remainder being starting material) of the monocarboxylic derivative (**1b**).

Amorphous solid. $R_f = 0.21$ (eluent AcOEt–MeOH–H_2O 8:4:1). ^{1}H NMR (D_2O) δ: 7.37 (2H, s, H-11 and H-12); 7.20 (1H, s, H-8); 6.97 (1H, s, H-4); 5.03 (1H, d, $J = 7.24$ Hz, H-1′); 4.49 (1H, dd, $J_1 = 6.25$ Hz, $J_2 = 11.9$ Hz, H-7); 3.97 (3H, s, CH_3O in C-2); 3.80 (1H, d, $J = 9.06$ Hz, H-5′); 3.63 (3H, s, CH_3O in C-1); 3.50–3.70 (3H, m, H-2′, H-3′ and H-4′); 2.67 (1H, dd, $J_1 = 5.65$ Hz, $J_2 = 12.8$ Hz, H-5a); 2.49 (3H, s, CH_3S); 2.25 (2H, m, H-6a and H-5b); 2.01 (3H, s, C*H*$_3$CO); 1.80 (1H, m, H-6b). ^{13}C NMR (D_2O) δ: 185.3 (C-6′); 177.7 (C-9); 176.1 (CONH); 161.5 (C-10); 155.0, 153.0 and 152.5 (C-1, C-3 and C-7a); 143.4 (C-2); 141.0 (C-12a); 138.5 (C-12); 137.9 (C-4a); 131.4 and 130.1 (C-8 and C-11); 129.4 (C-12b); 114.1 (C-4); 102.7 (C-1′); 78.8, 78.0, 75.4 and 74.3 (C-2′, C-3′, C-4′ and C-5′); 64.5 (CH_3O in C-2); 64.1 (CH_3O in C-1); 55.2 (C-7); 37.5 (C-6); 31.2 (C-5); 24.2 (*CH*$_3$CONH); 16.7 (SCH_3). FT-ICR: 622.13213 Da $[M–H + 2Na]^+$ (theoretic: 622.13295 Da).

2.7. Oxidation of colchicoside (**2**) with T. pubescens laccase

Colchicoside (**2**, 154 mg, 0.28 mmol) was dissolved in 10 mL acetate buffer, 20 mM and pH 4.5. TEMPO (11.3 mg, 0.07 mmol) and 1 mL of enzymatic solution (24 U) were added and the reaction was gently shaken at RT for 7 days, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 8:4:1). The solvent was evaporated and the residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 8:4:1) to give 105 mg (0.19 mmol, 66% yield, the remainder being starting material) of the monocarboxylic derivative (**2a**).

Amorphous solid. $R_f = 0.18$ (eluent AcOEt–MeOH–H_2O 8:4:1). ^{1}H NMR (D_2O) δ: 7.42 (1H, d, $J = 11.6$ Hz, H-12); 7.40 (1H, s, H-8); 7.22 (1H, d, $J = 11.1$ Hz, H-11); 6.97 (1H, s, H-4); 5.03 (1H, d, $J = 7.36$ Hz, H-1′); 4.49 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 11.8$ Hz, H-7); 4.02 (3H, s, CH_3O in C-10); 3.97 (3H, s, CH_3O in C-2); 3.80 (1H, d, $J = 9.10$ Hz, H-5′); 3.62 (3H, s, CH_3O in C-1); 3.50–3.70 (3H, H-2′, H-3′ and H-4′); 2.66 (1H, dd, $J_1 = 6.6$ Hz, $J_2 = 12.9$ Hz, H-5a); 2.27 (2H, m, H-6a and H-5b); 2.01 (3H, s, *CH*$_3$-CONH); 1.90 (1H, m, H-6b). ^{13}C NMR (D_2O) δ: 181.8 (C-6′); 177.4 (C-9); 173.5 (NHCO); 166.4 (C-10); 155.2 (C-1); 153.8 (C-3); 153.2 (C-7a); 144.5 (C-2); 139.3 (C-12a); 138.6 (C-12); 136.9 (C-4a); 132.1 (C-8); 129.7 (C-12b); 115.9 (C-11); 114.4 (C-4); 103.7 (C-1′); 78.8, 75.7 and 74.4 (C-2′, C-3′ and C-4′); 77.4 (C-5′); 63.2 (CH_3O in C-2); 62.8 (CH_3O in C-1); 57.9 (CH_3O in C-10); 54.8 (C-7); 37.9 (C-6); 31.2 (C-5); 23.3 (*CH*$_3$CO). FT-ICR: 606.15322 Da $[M–H + 2Na]^+$ (theoretic: 606.15579 Da).

2.8. Oxidation of amygdalin (**3**) with T. pubescens laccase

Amygdalin (**3**, 100 mg, 0.22 mmol [7]) was dissolved in 10 mL acetate buffer, 20 mM and pH 4.5. TEMPO (10 mg, 0.06 mmol) and 1.0 mL of enzymatic solution (54 U) were added and the reaction was gently shaken at RT for 48 h, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 8:2:1). The solvent was evaporated and the residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 8:2:1 and then 7:3:1) to give 48 mg (0.10 mmol, 47% yield, the remainder being starting material) of the monocarboxylic derivative (**3a**).

Amorphous solid. $R_f = 0.10$ (eluent AcOEt–MeOH–H_2O 8:2:1). ^{1}H NMR (CD_3OD) δ: 7.64 (2H, m) and 7.45 (3H, m)—ArH; 5.92 (1H, s, C*H*CN); 4.59 (1H, d, $J = 7.8$ Hz, H-1′); 4.37 (1H, d, $J = 7.5$ Hz, H-1); 4.26 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 2.0$ Hz, H-6b); 3.87 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 6.8$ Hz, H-6a); 3.67 (1H, d, $J = 9.3$ Hz, H-5′); 3.3 (3H, m); 3.5 (4H, m). ^{13}C NMR (CD_3OD) δ: 175.7 (C-6′); 133.8, 129.4, 128.7 and 127.4 (Ar); 118.3 (*C*N); 103.5 (C-1′); 101.5 (C-1); 74.5 (C-5′); 76.4, 76.3, 73.6, 73.4, 72.3 and 70.3 (C-2, C-3, C-4, C-5, C-2′, C-3′ and C-4′); 68.7 (C-6); 67.6 (*C*HCN). FT-ICR: 516.10747 Da $[M–H + 2Na]^+$ (theoretic: 516.10884 Da) and 1009.23100 Da $[2M–2H + 3Na]^+$ (theoretic: 1009.22846 Da).

2.9. Oxidation of asiaticoside (**4**) with T. pubescens laccase

Asiaticoside (**4**, 101 mg, 0.11 mmol) [9] was dissolved in 15 mL acetone and 35 mL acetate buffer, 20 mM and pH 4.5. TEMPO (3.5 mg, 0.02 mmol) and 2 mL of enzymatic solution (52 U) were added and the reaction was gently shaken at RT for 7 days, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 10:3:1). The solvent was evaporated and the residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 10:3:1) to give 25 mg (0.026 mmol, 24.4% yield, the remainder being starting material) of the monocarboxylic derivative **4a**.

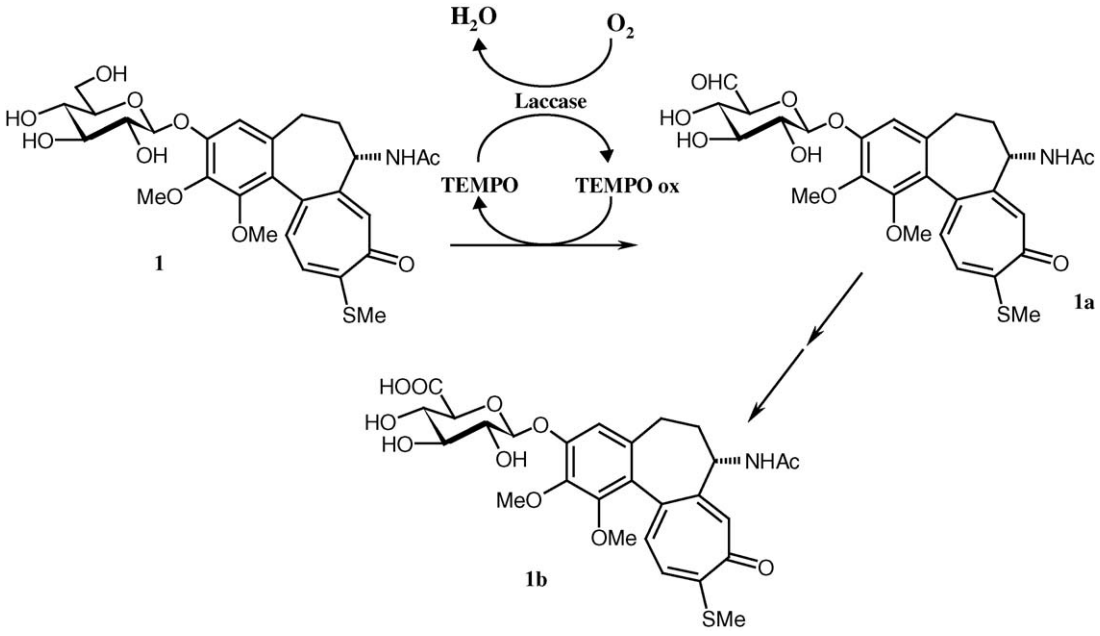
Amorphous solid. $R_f = 0.13$ (eluent AcOEt–MeOH–H_2O 10:3:1). ^{1}H NMR (CD_3OD) selected data δ: 5.33 (1H, d, $J = 8.0$ Hz, H-1′); 5.28 (1H, br t, $J = 3.4$ Hz, H-12); 4.81 (1H, d, $J = 1.0$ Hz, H-1‴); 4.37 (1H, d, $J = 7.7$ Hz, H-1″); 4.11 (1H, dd, $J_1 = 10.2$ Hz, $J_2 = 1.8$ Hz, H-6′); 4.01 (1H, dq, $J_1 = 8.5$ Hz, $J_2 = 7.0$ Hz, H-5‴); 3.88 (br dd, 1H, H-2‴); 3.75 (1H, dd, $J_1 = 10.2$ Hz, $J_2 = 4.8$ Hz, H-6′$_b$); 3.52 (1H, d, $J = 9.5$ Hz, H-5″); 2.26 (1H, d, $J = 11.3$ Hz, H-18); 1.27 (3H, d, $J = 6.2$ Hz, CH_3-6‴); 1.15 (3H, s, CH_3-27); 1.07 (3H, s, CH_3-25); 0.99 (3H, br s, CH_3-30); 0.91 (3H, d, $J = 6.5$ Hz, CH_3-29); 0.85 (3H, s, CH_3-26); 0.72 (3H, s, CH_3-24). ^{13}C NMR (CD_3OD) δ: 178.9 (C-28); 140.3 (C-13); 128.0 (C-12); 105.6 (C-1″); 103.6 (C-1‴); 96.7 (C-1′); 82.4 (C-4″); 79.6 (C-5′); 79.1 (C-3); 78.7 (C-5″); 77.7 (C-3″); 76.1 (C-3′ and C-2″); 75.0 and 74.8 (C-2′ and C-4‴); 73.1 (C-2‴ and C-3‴); 72.4 (C-4′); 71.2 (C-5‴); 70.8 (C-6′); 70.6 (C-2); 67.8 (C-23); 55.1 (C-18); 45.0 (C-4); 44.4 (C-14); 42.0 (C-8); 41.3 (C-20); 41.1 (C-19); 40.0 (C-10); 38.5 (C-22); 34.6 (C-7); 32.6 (C-21); 30.3 (C-15); 26.2 (C-16); 25.5 (C-11); 25.0 (C-27); 22.4 (C-30); 20.1 (C-6); 19.0 (C-26); 18.8 (C-25); 18.5 (C-6‴ and C-29); 14.7 (C-24). FT-ICR: 1017.45970 Da $[M–H + 2Na]^+$ (theoretic: 1017.46416 Da).

2.10. Oxidation of ginsenoside R_E (**5**) with T. pubescens laccase

Ginsenoside R_E (**5**, 151 mg, 0.16 mmol) was dissolved in 1.6 mL DMF and 15 mL acetate buffer, 20 mM and pH 4.5. TEMPO (16.8 mg, 0.11 mmol) and 1 mL of enzymatic solution (24 U) were added and the reaction was gently shaken at RT for 4 days, monitoring the conversion by TLC (eluent AcOEt–MeOH–H_2O 10:3:1). Two spots, with R_F 0.21 and 0.08, were detected. The solvent was evaporated and the residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O 10:3:1) to give 23 mg (0.025 mmol, 15% yields) of the compounds with R_F 0.21, which proved to be a 2:1 mixture of the two isomeric monocarboxylic derivatives (**5a** and **5b**) by NMR analysis. The more polar product, probably the dicarboxylate derivative **5c**, could not be isolated.

2.10.1. *5a* and *5b*

Amorphous solid. $R_f = 0.21$ (eluent AcOEt–MeOH–H_2O 10:3:1); FT-ICR: 983.50293 Da $[M + Na]^+$ (theoretic: 983.51916 Da). ^{1}H NMR (CD_3OD) selected data δ: 5.33 (1H, s, H-1″); 4.67 and 4.61 (2d, $J = 6.9$ and 8.0 Hz, H-1′ and H-1‴ of the main product); 4.66 and 4.62 (2d, $J = 7.8$ and 7.2 Hz, H-1′ and H-1‴ of the minor product). ^{13}C NMR (CD_3OD) selected data δ: 176.1 (COOH), 62.66 and 62.11 (residual CH_2-6′ and CH_2-6‴ in a 2:1 ratio).



Scheme 1. Laccase–TEMPO-mediated oxidation of thiocolchicoside (**1**).

3. Results and discussion

Thiocolchicoside (**1**) is a semi-synthetic alkaloid, easily obtained from its natural precursor colchicoside (**2**) through CH_3SNa treatment. Both these compounds have been claimed to be significantly less toxic than the aglycon colchicine, still maintaining a therapeutic action against human gout and other inflammations [10].

Laccase-mediated oxidation of **1** was attempted following standard reaction conditions (Scheme 1) [7]: *T. pubescens* laccase (2.4 U/mL) was added to a 27 mM solution of **1** in 10 mL 20 mM acetate buffer, pH 4.5, containing 0.1 eq of TEMPO, and the solution was gently shaken at RT for 8 days. TLC analysis showed the formation of a transient less polar compound **1a** (eluent AcOEt–MeOH–H_2O 8:4:1, R_f **1a**, 0.52, R_f **1**, 0.48) and of a main more polar product **1b** with R_f 0.21. Quite unexpectedly, silica gel chromatography failed to isolate the less polar product **1a**, whereas the main product was obtained and characterized as the expected glucopyranosidurinate **1b** by mass spectrometry and NMR analysis. The ^{13}C NMR spectrum of **1b** showed the disappearance of the signal due to the C-6′ CH_2OH at 61.0 ppm and the formation of a new signal at 185.3 ppm, diagnostic for the presence of a new COOH group. Similarly, in comparison to **1**, the ^1H NMR spectrum of **1b** was lacking of the signals due to CH_2-6′ (originally present as a dd at 3.53 ppm, $J_1 = 12.6$ and $J_2 = 5.25$ Hz, and a dd at 3.77 ppm, $J_1 = 12.6$ and $J_2 = 2.1$ Hz), while the signal due to CH-5′ became a simplified doublet downfield-shifted at 3.80 ppm ($J = 9.06$ Hz). Finally, the FT-ICR spectrum showed a quasi-molecular ion at 622 Da, thus confirming the proposed structure for **1b**.

In order to get information on the chemical structure of the less polar intermediate, the reaction progress was monitored by

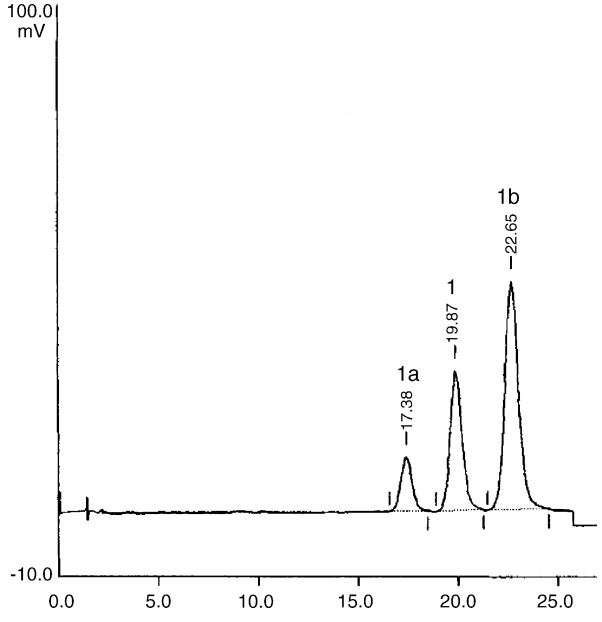


Fig. 1. Typical HPLC chromatogram of a laccase–TEMPO-mediated oxidation of thiocolchicoside (**1**).

Table 1
Percentage of conversion of thiocolchicoside (**1**) into **1a** and **1b** as a function of TEMPO concentration

Reaction time (days)	TEMPO concentration[a]			
	0.01	0.05	0.1	0.2
1	0	7.3	20.7	39.2
2	0.5	17.9	37.8	57.7
3	1.1	26.4	47.8	66.6
4	1.3	34.3	55.6	75.3
7	3.2	43.9	65.2	83.8

[a] mol/mol vs. thiocolchicoside (**1**).

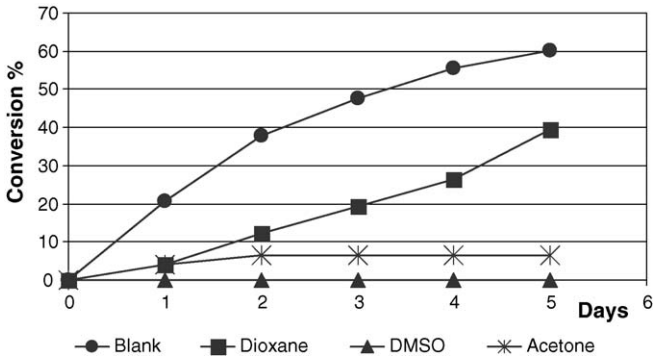


Fig. 2. Laccase–TEMPO-mediated oxidation of thiocolchicoside (**1**) in the presence of 20% (v/v) water-miscible cosolvents.

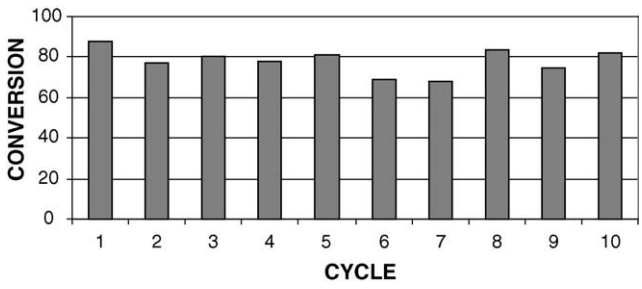
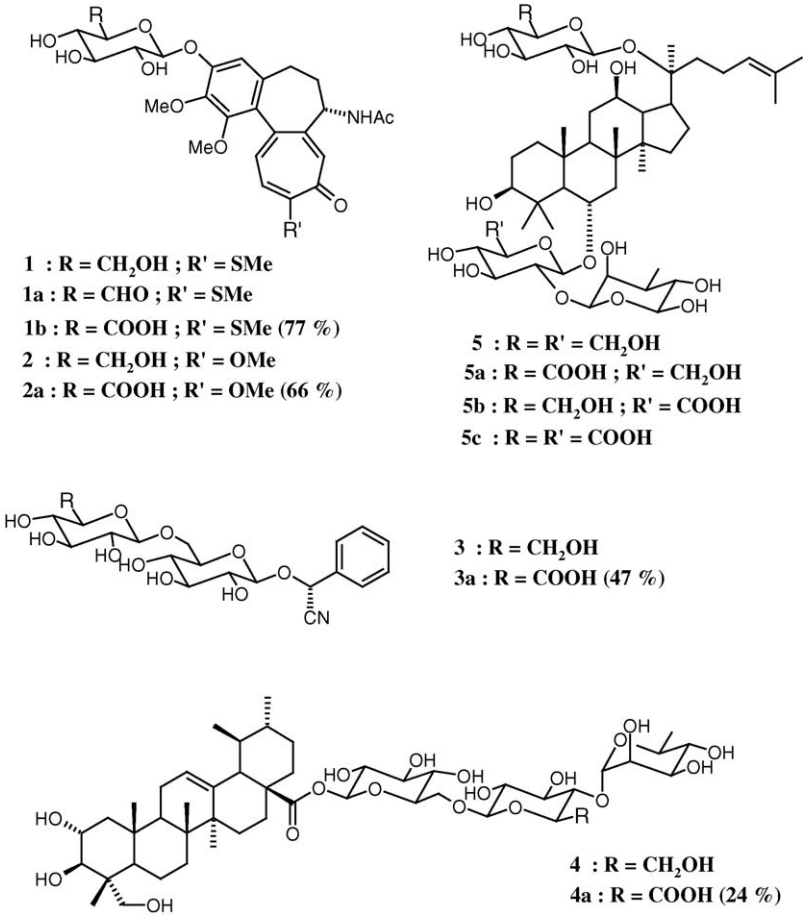


Fig. 3. Degrees of conversion of **1** after 24 h, using the same preparation of immobilized *Trametes pubescens* laccase.

HPLC on a reverse phase C-18 column. Fig. 1 shows a typical HPLC chromatogram of the reaction mixture. The most retained peaks were easily identified as **1** and **1b** (R_t 19.87 and 22.65 min, respectively) by comparison with authentic samples. The identity of the less retained peak (R_t 17.38) was assessed by HPLC–MS analysis: the compound had a M + Na molecular peak of 618 Da, corresponding to the hydrated form of the expected aldehyde **1a**. As previously observed with other sugar derivatives [7], the aldehydic intermediate was not stable under these reaction conditions and suffered additional oxidation to give the corresponding glucuronate. It is worth noting that the thiol group on the aglycon moiety was insensitive to the laccase-mediated oxidation, and the formation of the corresponding sulfoxides (or sulfone) derivatives was never observed.

Having in hands an efficient analytical method to quantitatively evaluate the reaction outcomes, we started our investigation on the optimization of the reaction conditions. As previously observed [7], TEMPO was by far the best chemical mediator in comparison with other compounds described in the literature (ABTS, HBT, VLA and HPI) [5]. Table 1 compares the degree of conversions of **1** after 7 days in the presence of different equivalent of TEMPO. Quite unexpectedly, we found that the higher the concentration of TEMPO, the higher the degree of oxida-



Scheme 2. Formulae of compounds **1**–**5**.

tion of **1** was: a clear indication that a fine-tuning of molar ratio between glycoside substrate, TEMPO and enzymes is required in order to define the optimal conditions for these biotransformations.

Thiocolchicoside is well soluble in water solutions, whereas most of the other natural glycosides are not, mainly due to the hydrophobic nature of their aglycons. In order to extend the applicability of this methodology to a wider group of compounds, we investigated the effect of water-miscible cosolvents on the laccase-mediated oxidation of thiocolchicoside. Reactions were performed in the presence of 20% (v/v) of different cosolvents, chosen among the ones that were shown not to significantly affect enzyme stability (see Section 2). As shown in Fig. 2, the degrees of conversion of **1** in the presence of 20% (v/v) dioxane or acetone were significantly lower than in the blank reaction, and, moreover, in the presence of 20% (v/v) DMSO the starting material was recovered unaffected. These data strongly indicated that organic cosolvents might influence not only the stability but also the activity of *T. pubescens* laccase, specifically when acting on TEMPO. The obvious conclusion is that organic cosolvents can be used to improve substrate solubility, but a preliminary investigation, finalized to evaluate their possible negative influence on laccase performances, is needed when using new substrates.

Oxidation of thiocolchicoside was then performed on a preparative scale and the corresponding glucopyranosidurinate **1b** was isolated in 77% yields. As a further contribution to the scaling up of these bio-oxidations, *T. pubescens* laccase was covalently linked to Eupergit C250L following a standard protocol. As shown in Fig. 3 (for details see Section 2), the recovered immobilized enzyme catalyzed several oxidative cycles, without showing significant loss of activity.

Finally, laccase–TEMPO-mediated oxidation has been extended to other water-soluble natural glycosides, colchicoside (**2**, Scheme 2) and amygdalin (**3**), and to other compounds that are almost insoluble in water, like asiaticoside (**4**) and ginsenoside R_E (**5**). With these two compounds the oxidations were performed in the presence of 20% (v/v) acetone and 10% (v/v) DMF, respectively, to allow their solubilisation in the reaction medium. The mono-oxidized derivatives **2a**–**4a** were isolated and characterized by NMR and mass spectrometry, while the presence of two primary OH's in the sugar moieties of **5** furnished the expected mixture of mono- and di-glucopyranosidurinates **5a**–**c**.

4. Conclusions

We have shown that the laccase–TEMPO system can be exploited for the regioselective oxidation of the primary OH's of natural glycosides. These biotransformations are performed in mildly acidic water solutions, and therefore this method is complementary to other chemical approaches for the in situ regeneration of the oxidized form of TEMPO, like sodium hypochlorite, that requires alkaline pH.

Additionally, it has been demonstrated that a careful choice of water-miscible cosolvents can allow the oxidation of poorly water-soluble glycosides and that the scaling-up of these biotransformations seems to be feasible, also thanks to the significant stability of the immobilized laccase. Future experiments will analyze in more details the influence of water-miscible cosolvents as well as of oxygen concentration on laccase activity and stability, and the results will be reported in due course.

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