Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 992 (2003) 85–100
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Use of liquid chromatography–diode-array detection and mass spectrometry for rapid product identification in biotechnological synthesis of a hydroxyprogesterone

Johan Lindholm[a], Douglas Westerlund[b], Karl-Erik Karlsson[c], Karin Caldwell[a], Torgny Fornstedt[a,*]

[a]*Center for Surface Biotechnology, Uppsala University, BMC Box 577, SE-751 23 Uppsala, Sweden*
[b]*Analytical Pharmaceutical Chemistry, Uppsala University, Uppsala, Sweden*
[c]*AstraZeneca R&D in Mölndal, Mölndal, Sweden*

Received 20 September 2002; received in revised form 22 January 2003; accepted 3 February 2003

Abstract

In exploratory scale biotechnological process development, the product must be rapidly identified although a reference compound may not always be available. LC–diode-array detection and MS were used for this purpose in a process producing 9α-hydroxyprogesterone from progesterone as substrate. The electrospray ionization mass spectrometer was combined with an ion trap mass spectrometer for the second generation MS. The preliminary identification, which could be carried out within the course of a day, confirmed that the product was a hydroxyprogesterone. The final identification step, which was much more material intensive and hence time consuming, involved a two-step preparative separation to yield quantities necessary for definitive product identification based on ^{1}H- and ^{13}C NMR.

Keywords: Preparative chromatography; Hydroxyprogesterones; Progesterones; Steroids

1. Introduction

An important step in the design of a biotechnological production protocol is to produce reference material of the product for further use in the optimization and validation of the process for the pilotscale (100–300 l) and the production-scale (~m^3) levels. The reference material is obtained in the laboratory-scale synthesis (~10 l size) which produces a sufficient quantity of material for unambiguous verification of its chemical identity by NMR. However, because the fermentation process is time-consuming (around 1 week), rapid analytical methods for the preliminary characterization of the formed product without a priori need of reference material are highly desired, with the objective to enable one to stop the process as quickly as possible if the production gives undesirable results. It was the goal of this study to investigate the use of HPLC–diode-array detection and advanced mass spectrometry to fulfil the purpose to provide rapid and preliminary identification of the

*Corresponding author. Tel.: +46-18-471-4879; fax: +46-18-555-016.
E-mail address: torgny.fornstedt@ytbioteknik.uu.se (T. Fornstedt).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00314-5

product without any reference material available. NMR was applied for the definitive identification.

DAD has been proposed for various applications, such as preliminary identification of a steroidal glycoside in seed [1], peptide mapping [2], assay of sulfamethazine in animal tissues [3] but has not been reported for biotechnological processes. Electrospray ionization (ESI) [4] has proved to be a most versatile tool for soft ionization of a large variety of analytes. Ion trap MS, in turn, is especially useful for advanced qualitative work due to the inherent ability of the technique to provide scans up to the MS^5 range; for a review see Ref. [5]. An overview of the use of LC– and GC–MS for the analysis of steroids was published recently [6].

As a model of a biotechnological process we used the production of 9α-hydroxyprogesterone using progesterone as substrate. 9α-Hydroxy steroids are useful intermediates in the production of a number of pharmaceutically active compounds [7,8]. We have recently [9] cloned a gene for steroid 9α-hydroxylase into *Escherichia coli* and expressed the enzyme in the presence of either progesterone (PS) or 4-androstene-3,17-dione (AD) as substrates.

2. Experimental

2.1. Chemicals and solvents

2.1.1. Fermentation process

LB is a chemically ill-defined complex medium containing 5 g/l of yeast extract (a source of nitrogen and water soluble B vitamins) and tryptone 10 g/l (pancreatic digest of casein) from Oxoid (Basingstoke, UK) and NaCl 10 g/l of reagent-grade quality from Merck (Darmstadt, Germany). M9 is a well-defined medium containing 30 g/l $Na_2HPO_4 \cdot 2H_2O$, 15 g/l KH_2PO_4, 2.5 g/l NaCl, 5 g/l NH_4Cl, 2 m*M* $MgSO_4$, 200 μ*M* $CaCl_2$, 0.4% (w/v) glucose and 1 ml of trace salts stock solution containing the following per litre: $CaCl_2 \cdot 6H_2O$ 0.74 g, $FeCl_2 \cdot 4H_2O$ 12.28 g, $ZnCl_2$ 0.094 g, $CuSO_4 \cdot 5H_2O$ 0.16 g, $MnSO_4 \cdot H_2O$ 0.113 g, $CoCl_2$ 0.1 g and Na-EDTA·$2H_2O$ 54 m*M*. All inorganic salts mentioned as well as glucose and sodium dihydrogen ethylenediaminetetraacetate (EDTA) were of analytical-reagent quality (except $CoCl_2$, purum) and were obtained from Merck. Riboflavin was from Calbiochem (La Jolla, CA, USA), kanamycin from Sigma (St Louis, MO, USA), carbenicillin from GibcoBRL (Burlington, Ontario, Canada), polypropylene glycol from BDH (Poole, UK) and IPTG from Diagnostic Chemicals (Oxford, CN, USA). NaOH, H_3PO_4 and NH_4OH were of analytical quality (Merck). The ethanol used was 99.5% pure and was obtained from Kemetyl (Haninge, Sweden).

2.1.2. Analytical processes

Progesterone (PS) minimum 99%, 4-androsten-3,17-dione (AD), 11α-hydroxyprogesterone (11α-OH-PS), 11β-hydroxyprogesterone (11β-OH-PS), 20α-hydroxyprogesterone (20α-OH-PS) and 20β-hydroxyprogesterone (20β-OH-PS) were all minimum 98% and obtained from Sigma (Stockholm, Sweden). 9α-Hydroxyprogesterone (9α-OH-PS) was obtained by fermentation followed by preparative HPLC, described in this article. The solution of the product for MS analysis was prepared from HPLC-purified material. The organic solvents used for chromatography, *n*-hexane, ethyl acetate, isopropanol, methyl *tert.*-butyl ether (MTBE) and acetonitrile, were of LiChrosolv-grade, from Merck and methanol was of HPLC Gradient Grade from J.T. Baker (Deventer, Netherlands). Methylene chloride used for extractions was of LiChrosolv-grade, from Merck. The water used in the mobile phase was obtained from the Milli-Q Academic Gradient A10 Biocel Synthesis system (Millipore, Sundbyberg, Sweden). All mobile phases were degassed before use.

2.2. Apparatus

2.2.1. LC–DAD system

All HPLC experiments were carried out with a Hewlett-Packard HP 1100 Chemstation with an auto injector, equipped with a valve-switching unit with 10 port valves, a built in diode-array UV detector and a personal computer (Agilent Technologies, Palo Alto, CA, USA). DAD made it possible to acquire spectra of all peaks in the chromatogram. The UV signals were recorded at 245 nm (bandwidth 4 nm, slit 2 nm), respectively. The on-line UV spectra and three-dimensional spectra chromatograms were recorded from 190 to 400 nm. The HPLC system was complemented with a fraction collector from Advan-

tec (Advantec Toyo Roshi International, Dublin, CA, USA).

2.2.2. HPLC columns

The column Kromasil KR100-3.5C_{18} (particle size: 3.5 μm; 150 mm×4.6 mm I.D) was used for analytical purposes with methanol–water. The chiral column Cyclobond I 2000 (particle size: 5 μm; 250 mm×4.6 mm I.D.) was obtained from Astec (Advanced Separation Technologies, Whippany, NJ, USA). Hypersil Hypurity Elite-C_{18} (pore size=180 Å; 5 μm; 100 mm×4.6 mm) was obtained from Hypersil (Cheshire, UK). Nucleosil C_{18} (5 μm; 100 mm×4.6 mm) was obtained from Macherey-Nagel (Düren, Germany). For all columns, the pore size is 100 Å, if not otherwise stated. The columns Kromasil KR100-7-SIL (particle size 7.0 μm; 250 mm×10.0 mm I.D.) and Kromasil KR100-5C_{18} (particle size: 5.0 μm; 250 mm×10.0 mm I.D.) were used for preparative purposes with *n*-hexane–ethyl acetate (10:90) and with methanol–water (80:20) as the mobile phases, respectively. All Kromasil columns were obtained from Eka Chemicals (Bohus, Sweden). All separations were carried out at ambient temperature.

2.2.3. Mass spectrometry

A MAT 900 double focusing mass spectrometer (ThermoFinnigan, Bremen, Germany) with electrospray ionisation was coupled to a Finnigan ion trap mass spectrometer for the detection of product ion spectra. Tuning and optimisation of the instrument were carried out by infusion of the analytes to obtain optimum settings and spray positioning for the selected mass transitions. All samples were dissolved in acetonitrile–2 m*M* aqueous ammonium acetate (50:50) and were infused continuously into the ESI source at a flow-rate of 1 μl/min. Spectra were obtained in the positive ion mode. Accurate mass measurement was done using two reference masses from polyethylene glycol 400 (Aldrich-Chemie, Steinheim, Germany). The parent ion was selected in the magnetic sector and the mass window for precursor selection was 1000 ppm. Relative collision energy was about 25%. For each analyte, the protonated molecule was isolated in the magnetic sector instrument and transferred to the ion trap. The collision gas in the ion trap was helium. The source voltage of the electrospray was 1 kV and the capillary temperature was 230 °C. Data were processed using Navigator (version 1.1) software.

2.2.4. NMR

^{1}H and ^{13}C NMR spectra were recorded for $C^{2}HCl_3$ solutions at 400 and 100.6 MHz, respectively with a Unity 400 NMR spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported in ppm referenced to tetramethylsilane via the solvent signal (^{1}H at 7.26, ^{13}C at 77.0). NMR signals were assigned from P.E.COSY [10], HSQC [11], HSBC [12], NOESY [13], ROESY [14] and NOE difference spectra [15]. For NOESY and ROESY experiments, the mixing time was set to 1 s. PFG enhanced versions of the NOESY, HSQC, and HMBC experiments were used [16].

2.3. Procedures

2.3.1. Preparation of analytical samples

A 1.0-ml volume of the aqueous process liquid was vortexed for 10 s with 1.5 ml methylene chloride. The mixtures were ultrasonicated for 30 min and mechanically shaken for 120 min followed by centrifugation at 1000 g during 10 min. A 1.0-ml volume of the organic phase was transferred to a new test-tube and evaporated under a stream of N_2 to dryness at 38 °C. The residue was re-dissolved in mobile phase and injected into the chromatographic system.

2.3.2. Preparation of preparative samples

From the LB culture, 6 l of the final fermentation liquid including the cells were extracted with 5 l methylene chloride. When extracting the M9 culture, the cells were first removed by centrifugation and the supernatant was extracted as above. Thus only the soluble fraction of the fermentate was extracted. The organic phase was evaporated with a rotary evaporator. The extract was re-dissolved in 30 ml mobile phase, composed of ethyl acetate–*n*-hexane (90:10), and filtered through a 0.45-μm filter (GHP Acrodisc) to remove any particles that could block the column.

2.3.3. Fermentation

The micro-organism used was *Escherichia coli* strain BL21 modified by the insertion of a gene for the enzyme steroid 9α-hydroxylase from *Mycobacterium smegmatis*. The biotransformation was carried out in a fermentor with a working volume of 10 l. The culture was agitated continuously and the minimum stirrer speed was 200 rpm. pH of the culture was kept at 7.2 by addition of acid (3 *M* H_3PO_4) and base (3 *M* NaOH). To decrease the foaming, polypropylene glycol (PPG) was added before inoculation (0.2 ml/l). Antibiotics were also added to the fermentations (kanamycin, 50 μg/ml, and carbenicillin, 100 μg/ml, dissolved in 20 ml water each). Expression of the enzyme was induced by addition of isopropylthiogalactoside (IPTG, 0.1 m*M*, dissolved in 1 ml 70% ethanol). Riboflavin (5 μg/ml in 40 ml water) was added prior to induction. At the same time, the substrate progesterone (200 μ*M*) was added, dissolved in 30 ml absolute ethanol. The culture was run for 6 days after induction. A 10-ml sample was taken out each day with a syringe through a septum in the fermentor wall near the bottom, close to the impeller. There were no measurements of the liquid volume in the reactor, nor of the added amounts of acid and base for pH control. Extra nutrition was supplied to the culture through a needle inserted in the headplate of the fermentor: to the LB culture, 200 ml of 4× concentrated LB (i.e. 40 g/l of tryptone and NaCl and 20 g/l of yeast extract) was added once a day. For the M9 culture, 200 ml of 20% (w/v) glucose was added 1, 2 and 4 days after induction. Further details on the strain and fermentation procedures are described elsewhere [9].

3. Results and discussion

3.1. Fermentation

The fermentation process was running for a total period of 7 days. An aliquot of the process liquid was taken for analysis at 24-h intervals, during the whole period. The process was carried out in two different culture media, a rich medium (LB) and a minimal medium (M9). The complex medium LB was used to produce the product analyzed by HPLC–DAD and MS and the minimal medium M9 was used for the final identification by NMR. LB is a standard microbiological medium, which contains yeast extract (hydrolysed dry yeast), tryptone (proteolytically digested animal proteins) and NaCl. The exact composition is not known. M9 on the other hand is a well-defined minimal medium containing mineral salts and glucose. On the rich medium, bacterial growth is normally faster because many metabolites can be found in the medium, and do not have to be synthesised de novo from the elements.

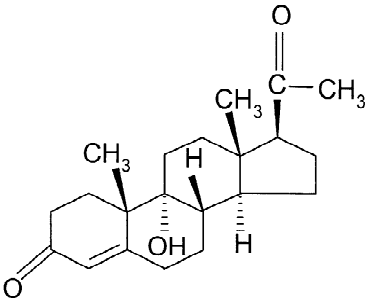
It was important to have a rapid and preliminary identification method combined with a preliminary quantitative monitoring. At that stage there is no time to purify the amounts of product required in order to make a proper NMR identification. LC–DAD and ESI-MS combined with ion-trap mass spectrometry was evaluated as for this preliminary product identification. The final identification was made using ^{1}H- and ^{13}C NMR.

3.2. Preliminary identification of the product using HPLC–DAD

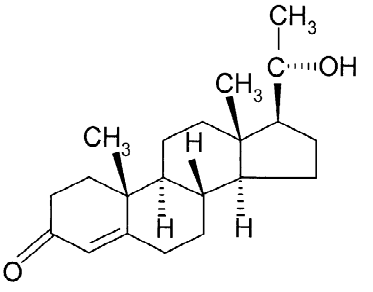
3.2.1. LC column selection

One important problem in this investigation is that the product is not available from any commercial distributor. Therefore, a sample mixture containing a number of compounds similar to the product (hydroxyprogesterones) was injected into the different columns in order to find the best system for resolving such components. The sample was a mixture of PS, 11α-OH-PS, 11β-OH-PS, 20α-OH-PS, 20β-OH-PS and AD, respectively (see Fig. 1 for the structures).

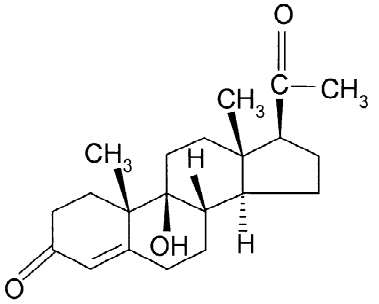
It is well known that many steroids, among them progesterone, are captured into and bounded in β-cyclodextrin [17,18]. The chiral phase Cyclobond I 2000 consisting of immobilized β-cyclodextrin is recommended for the separation of steroids and hydroxy steroids [19]. Cyclobond I 2000 was therefore tested using different mobile phases and the best mobile phase was found to be *n*-hexane–isopropanol (85:15). When the mixture containing the six steroids was injected, however, only five of the six steroid components appeared as peaks in the chromatogram. This was because the steroids 20α-OH-PS and 20β-OH-PS, had a combined elution (Table 1). In addition, the AD peak was too broad and deformed



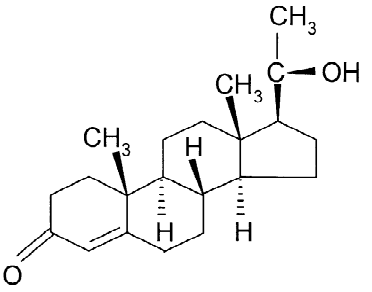
9α-hydroxyprogesterone (9α-OH-PS)
Mw: 330.46



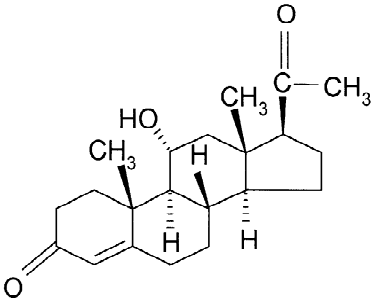
20α-hydroxyprogesterone (20α-OH-PS)
Mw: 316.48



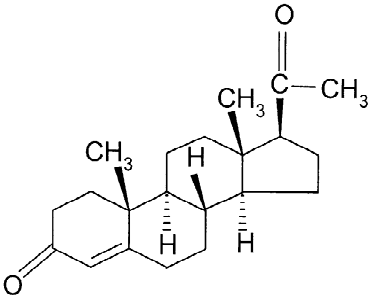
9β-hydroxyprogesterone (9β-OH-PS)
Mw: 330.46



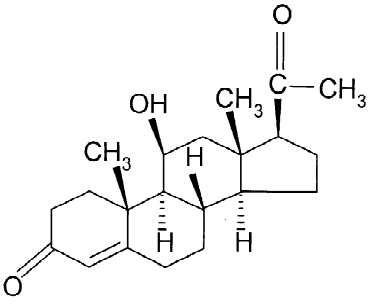
20β-hydroxyprogesterone (20β-OH-PS)
Mw: 316.48



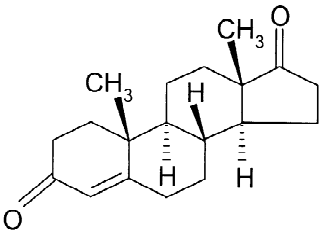
11α-hydroxyprogesterone (11α-OH-PS)
Mw: 330.46



Progesterone (PS)
Mw: 314.46



11β-hydroxyprogesterone (11β-OH-PS)
Mw: 330.46



4-androsten-3,17-dione (AD)
Mw: 286.41

Fig. 1. The structures and molecular masses (Mw) of the steroids 9α-OH-PS, 9β-OH-PS, 11α-OH-PS, 11β-OH-PS, 20α-OH-PS, 20β-OH-PS, PS, and AD.

Table 1
Comparison of retention times (t_R min), resolution (R_S), efficiency (N) and reduced plate height (h) for steroids using different phase systems

Phase system	Column dimensions (mm)	Analyte	t_R (min)	R_S	N	h
Cyclobond I 2000 5 μm, *n*-hexane–isopropanol (85:15)	250×4.6	PS	11.2		14 474	3.5
		20β-OH-PS	13.6	5.0	14 589	3.4
		20α-OH-PS	13.7	<0.4	10 862	4.6
		AD	20.7	1.3	455[a]	109.9[a]
		11β-OH-PS	25.9	1.7	9645	5.2
		11α-OH-PS	41.9	8.5	6468	7.7
Hypersil Hypurity C_{18} 5 μm, acetonitrile–water (50:50)	100×4.6	11α-OH-PS	3.2		1339	15.0
		11β–OH-PS	4.6	3.4	1748	11.4
		AD	5.1	1.0	1721	11.6
		20α-OH-PS	7.4	4.0	2016	9.9
		20β-OH-PS	10.6	3.7	1514	13.2
		PS	11.4	0.7	1881	10.6
Nucleosil C_{18} 5 μm, acetonitrile–water (50:50)	100×4.6	11α-OH-PS	4.6		5675	3.5
		11β-OH-PS	7.5	9.1	6436	3.1
		AD	8.5	2.6	6627	3.0
		20α-OH-PS	13.7	9.4	6468	3.0
		20β-OH-PS	18.9	6.5	6699	3.0
		PS	20.5	1.7	6633	3.0
Kromasil C_{18} 3.5 μm, acetonitrile–water (55:45)	150×4.6	11α-OH-PS	4.7		12 648	3.4
		11β-OH-PS	7.2	12.9	18 013	2.4
		AD	8.2	4.1	19 217	2.2
		20α-OH-PS	11.3	11.5	20 417	2.1
		20β-OH-PS	17.4	15.4	21 470	2.0
		PS	18.8	2.8	22 722	1.9

[a] The peak is extremely broad and deformed.

for calculating the plate height (cf. Table 1). Another disadvantage was the large difference in retention times between the substrate (PS) and some hydroxyprogesterones; in this system PS had the retention time 11.2 min while the retention of 11α-OH-PS was 41.9 min (cf. Table 1).

The newly developed chiral phase Kromasil KR100-CHI-TBB (not included in Table 1) consists of a network polymer with an incorporating bifunctional C_2-symmetric chiral selector; it was tested using different mobile phases. The mobile phase composed of *n*-hexane–MTBE (60:40) worked best. However, the column was not able to separate the steroids 20β-OH-PS and AD. In addition, all peaks were generally broad resulting in low efficiencies and high reduced plate heights. Since none of the two chiral phases gave especially good separations, the further investigation was made on less expensive C_{18} columns.

Several C_{18} reversed-phase systems with different methanol–water mixtures as mobile phases were tested. None of the 100 mm long C_{18} columns could separate 20α-OH-PS and PS from each other. The most expensive column, Hypersil Hypurity Elite column, gave the poorest efficiency (largest h) and poorest resolution (Table 1). An advantage was that the differences in retention times between progesterone and the hydroxyprogesterones were small keeping the analysis time short. A Nucleosil C_{18} column with 5-μm particles had much lower reduced plate height and resolution (lower h and larger R_s) compared to the more expensive Hypersil column (cf. Table 1). However, Kromasil KR100 with 3.5-μm particles had the lowest reduced plate height and best

resolution of all columns tested; the average *h* was 2.3 to be compared with 3.1 for Nucleosil and 12.0 for Hypersil, respectively (cf. Table 1). The Kromasil KR100 had also the best resolution with an average R_s value of 9.3 to be compared with 5.9 for Nucleosil and 2.6 for Hypersil, respectively (cf. Table 1). The average efficiency expressed as number of plates for the 150 mm Kromasil column was N_{av}=19 081. Since the Kromasil C_{18} 3.5 μm column had the best resolving properties and was chosen for the LC–DAD studies. However, due to environmental reasons, it was used with methanol as the organic modifier.

3.2.2. Spectral characteristics of the product

The evaporated extract of the real process liquid was dissolved in mobile phase and injected into the analytical Kromasil C_{18} column using as mobile phase a methanol–water mixture (70:30). A modern diode array detector was used which made it possible to obtain spectra of all peaks in the chromatogram. The on-line UV spectra and three-dimensional chromatograms of the steroid mixture were recorded from 190 to 400 nm. The steroid mixture (above) contained four hydroxyprogesterones—two α/β isomeric pairs, PS and AD. A preliminary acquisition of DAD spectra revealed that all hydroxyprogesterones had very similar spectral characteristics to progesterone. The software of the DAD instrument had a tool to compare acquired spectra with a so-called matching factor. The maximum value possible to obtain is 1000 but a matching factor of already 950 strongly indicates that the compound matched is identical with the reference compound. When the spectra of the four hydroxy steroids were matched versus the spectrum of progesterone the matching factor was not less than 990; thus their spectra strongly overlap with each other and with the substrate progesterone. The matching factor of AD versus PS was somewhat lower, however, close to 990. This is a very strong indication that the product 9α-hydroxyprogesterone (9α-OH-PS), not available at the time for this screening, should have the same spectrum as the substrate progesterone.

The reason for the great similarities of the spectra among the hydroxyprogesterones is because they all have the same UV absorbing element at the same position, i.e., a double bond conjugated to a carbonyl group (cf. Fig. 1). The characteristic UV absorption spectrum at the 240–250 nm is the result of $\pi \rightarrow \pi^*$ transitions in the conjugated α,β-unsaturated ketone at position 3 [20]. This results in absorption maximum for the steroids at approximately 245 nm. The spectra of the hydroxyprogesterones and of progesterone were all saved in the library of the DAD system in order to allow for the preliminary identification of the fermentation product. The on-line DAD signal was set to the absorption maximum of the progesterone and its derivatives, at 245 nm.

3.2.3. Three-dimensional DAD chromatograms

The resulting single wavelength detection (pilot-signal at 245 nm) chromatogram after injection of a worked-up sample of the process liquid at time zero (i.e., the start of the fermentation process) is shown in Fig. 2a. Only one single peak appears, the substrate peak. The corresponding full spectral three-dimensional chromatogram is shown in Fig. 2b; this has the spectral characteristics of progesterone. Fig. 3a shows the single wavelength chromatogram obtained after injection of a worked-up sample taken at the end of the fermentation process. Two additional peaks appeared of comparable height and similar retention times; they were less retained than the substrate. Since 9α-hydroxyprogesterone is less hydrophobic than the substrate, any of the peaks might correspond to the product, but it is not possible to assign the correct peak by comparing only retention times. This is further supported by comparing the retention of similar compounds made in a parallel study [21]. However, with the use of the full spectral three-dimensional chromatogram after the injection of the sample taken at the end of the fermentation process (cf. Fig. 3b) it was possible to decide which one might be the desired product, i.e., hydroxyprogesterone. It is clearly seen in Fig. 3b that only the new peak with the retention time of 4.5 min has a spectrum similar to the substrate. The earlier peak with retention of 3.0 min seems to have a spectrum more characteristic for aromatic structures. The spectrum of the suspected product peak was matched to the spectrum of the hydroxyprogesterones stored in the library. The library search showed a matching factor of about 990, which is indicative of a very large spectral overlap. This three-dimensional chromatogram shows also that the sample is essentially

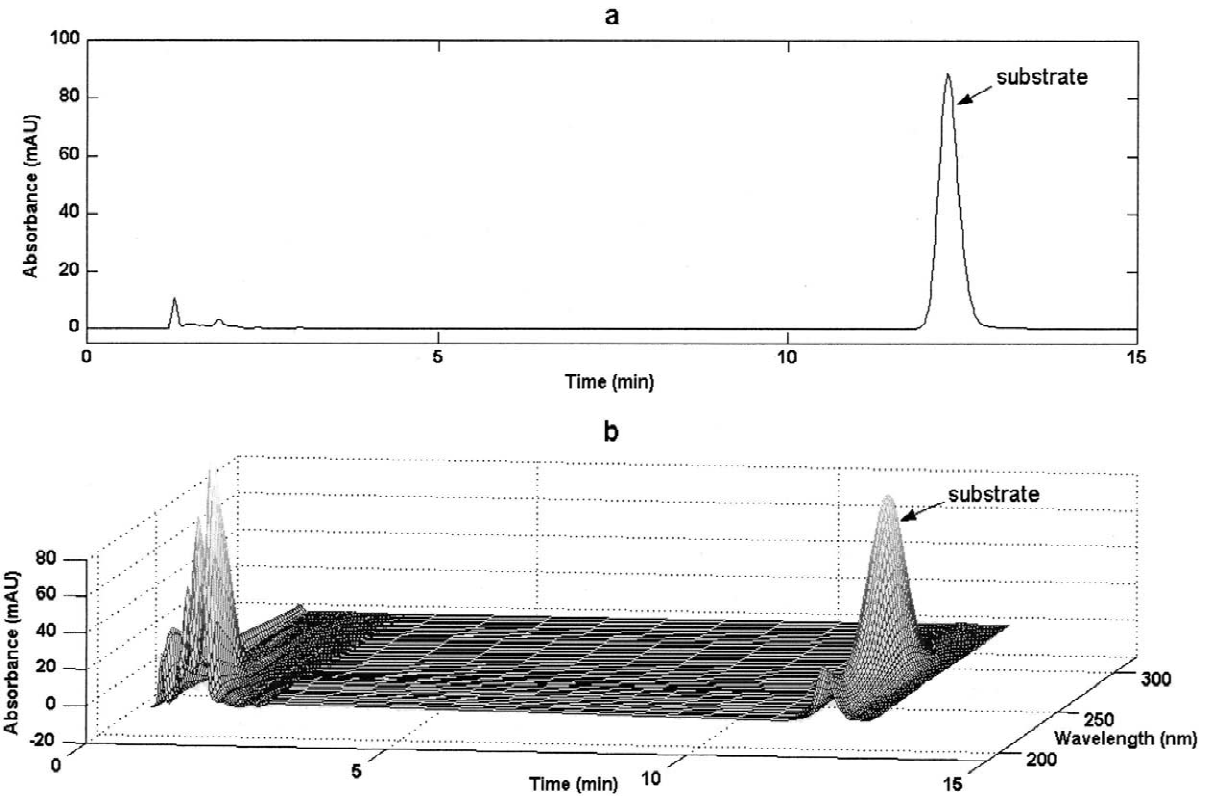


Fig. 2. (a) Chromatogram obtained from the injection of 20 μl extract from the beginning of the fermentation. The chromatogram displays only one peak, that of the substrate progesterone at 12.3 min. Chromatographic conditions were HP1100, column: Kromasil KR100-3.5-C_{18} (100×4.6 mm I.D.); mobile phase: methanol–water (70:30); flow-rate: 0.70 ml/min; UV absorbance detection at 245 nm. (b) Three-dimensional spectrochromatogram from the same injection as (a). The three-dimensional spectra of progesterone show the spectral characteristics of a steroid (see text).

free of other substances, with the exception of the peak eluting at around 2.6 min.

The results described above illustrate the high potential of HPLC–DAD systems to be used for process control of biotechnological processes. A simple liquid–liquid extraction sample preparation followed by a reasonably rapid separation provides a quick preliminary identification of the product. Such an analysis can be carried out as soon as the synthesis has started and the process can be stopped immediately if the LC–DAD analysis gives undesirable results.

3.3. High-resolution accurate mass determination and ESI ion trap MS

The LC–DAD identification was complemented with a high-resolution accurate mass determination using an electrospray ionization-magnetic sector MS system–ion trap mass spectrometer. For product ion spectra, the protonated molecular ion was selected in the magnetic sector instrument and then transferred to the ion-trap MS system for further analysis. The principles of ion trap MS mean that several generations of daughter ions can be generated providing multiple MS (MS^n) spectra. This capability provides a rich source of structural information. A common mass spectrometric approach for steroid identification is to compare the first- and second-generation product ion spectra of known reference compounds with the spectra obtained for the unknown sample. In this study, up to MS^5 spectra were investigated for different steroids.

3.3.1. Full scan spectra

Because the product 9α-OH-PS was not available

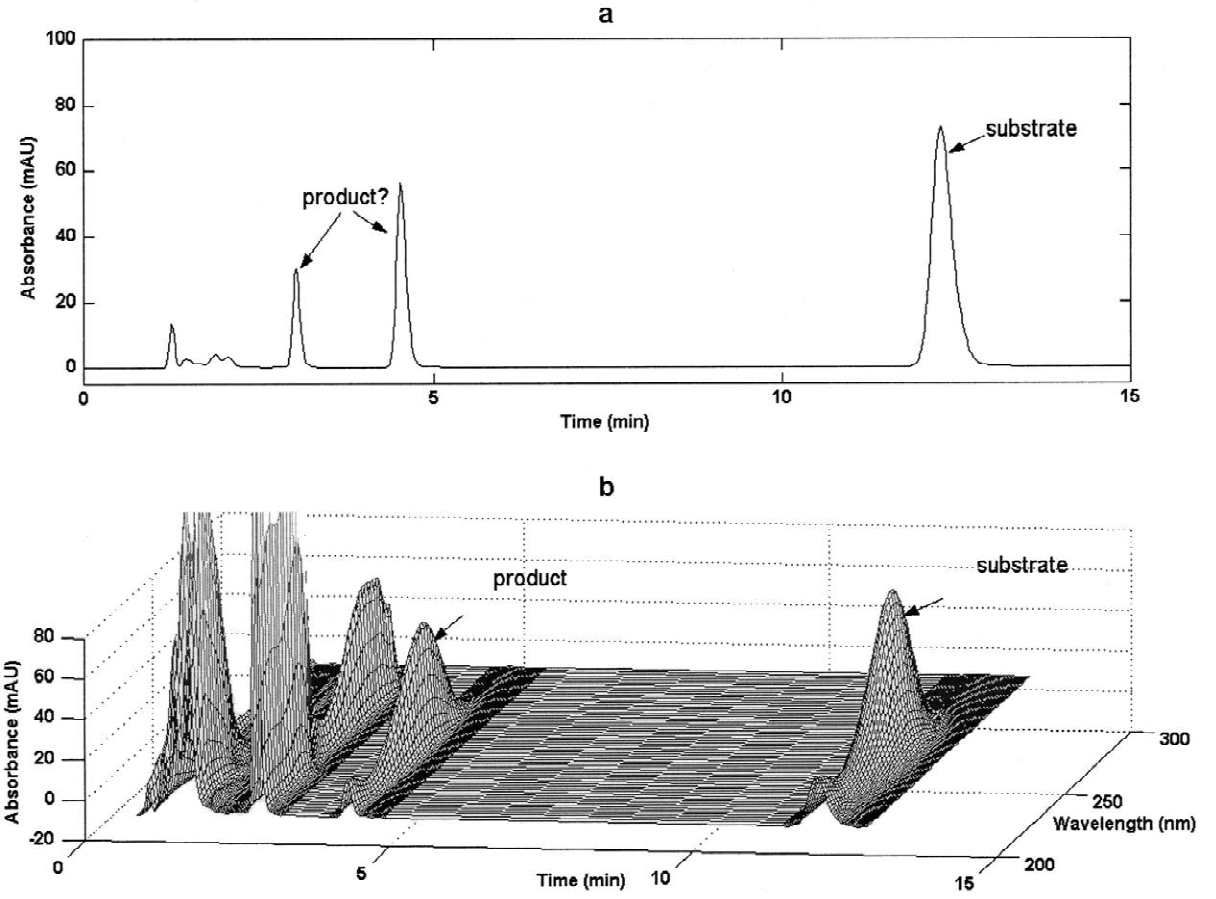


Fig. 3. (a) Chromatogram obtained from the injection of 20 μl extract from the end of the fermentation process. The chromatogram shows that two new peaks have appeared. The experimental conditions were as described under (a). (b) Three-dimensional spectrochromatogram from the same injection as (a). Only one of the two new peaks has the spectral characteristics of a steroid.

as a reference substance, full scan spectra were acquired using 11α-hydroxyprogesterone (11α-OH-PS) as a reference. Fig. 4a–c shows the full scan mass spectra of PS, 11α-OH-PS and the fermentation product, respectively. In this system, quasi-molecular ions $[M+H]^+$ appear as base peaks for PS (m/z 315.2), 11α-OH-PS (m/z 331.3) and for the fermentation product (m/z 331.3). This shows that the fermentation product has the same molecular mass as a hydroxyprogesterone, which very strongly indicates that the product really has such a structure. The spectrum of PS in Fig. 4a shows an extra peak at m/z 356.3, which could be an acetonitrile (ACN) adduct $[M+ACN+H]^+$. Such adducts are often formed when using a mobile phase containing acetonitrile [22] even when using nanospray. The intensity of the adducts is not decreased when increasing the electrospray voltage; instead the temperature of the ''heated capillary'' should be increased [23]. The spectra of 11α-OH-PS (Fig. 4b) and the fermentation product (Fig. 4c) show a fragment ion at m/z 313.4 due to loss of one water molecule, and a fragment ion at m/z 295.4 due to loss of two water molecules from the parent ion (m/z 331.3). The spectrum of the fermentation product also exhibits an ion at m/z 372.3, probably from $[M+ACN+H]^+$.

3.3.2. Epimer identification by relative ion intensities

It was suggested by Zaretskii et al. that it is possible from pure mass spectral data from electron impact ionization to decide the epimeric configura-

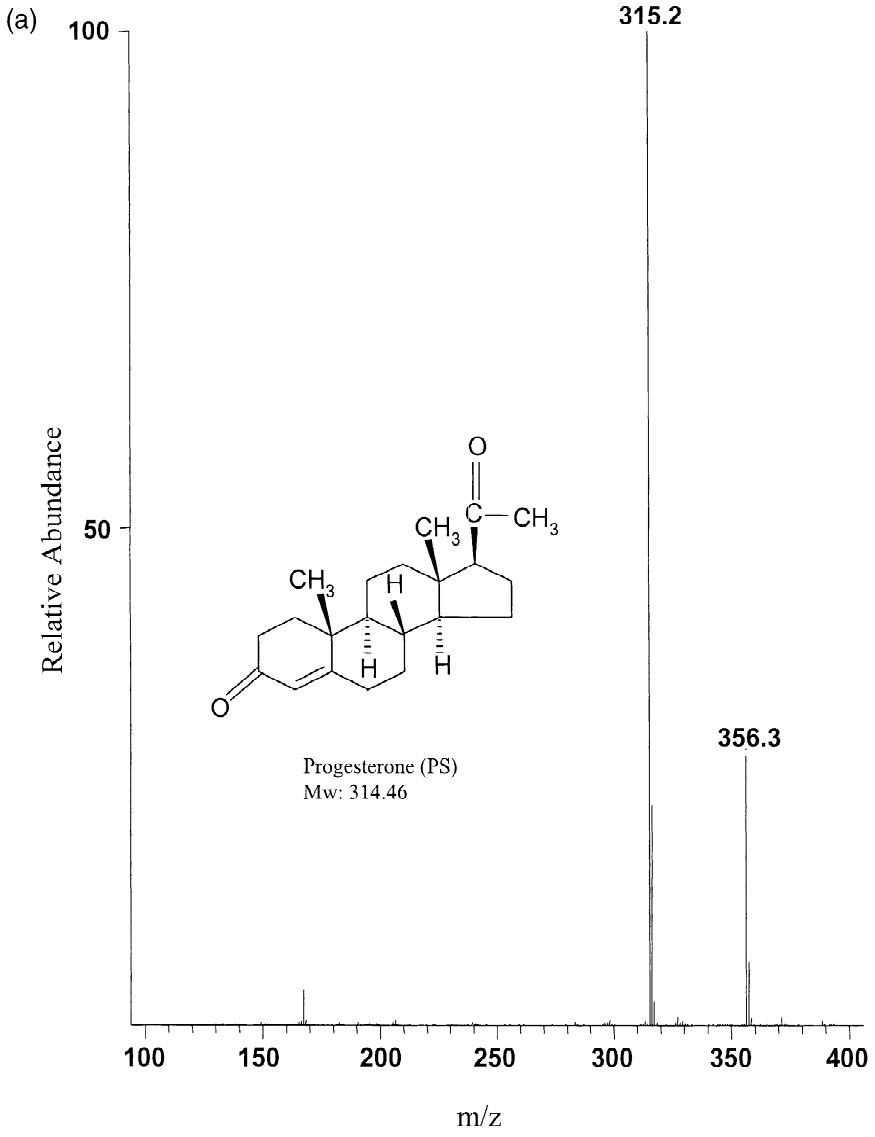


Fig. 4. Full-scan spectra of: (a) PS, from 100 to 400 u resulting from infusion of a 100 μ*M* standard solution; (b) 11α-OH-PS from 100 to 400 u resulting from infusion of a 100 μ*M* standard solution; (c) fermentation product, from 100 to 500 u resulting from infusion of a standard of 40 μg/ml of the product. All samples were dissolved in acetonitrile–2 m*M* aqueous ammonium acetate (50:50).

tion of the secondary alcohols of monohydroxy derivatives of progesterone [24]. Observed differences in relative intensities show that some hydroxyprogesterones readily eliminate water molecules, while other hydroxy steroids are more stable and dehydrate less readily. According to Zaretskii et al. [24], these differences should be a basis for determining whether the steroid has the hydroxyl group in an axial or an equatorial position. Zaretskii et al. [24] found that under electron impact ionization conditions axial hydroxyls on hydroxy steroids dehydrate more readily compared to equatorial hydroxyls. According to Fig. 1, the monohydroxyl group of 11α-OH-PS is equatorial and the tertiary hydroxyl group of the product 9α-OH-PS has an axial position. Thus the hydroxyprogesterone with an axial

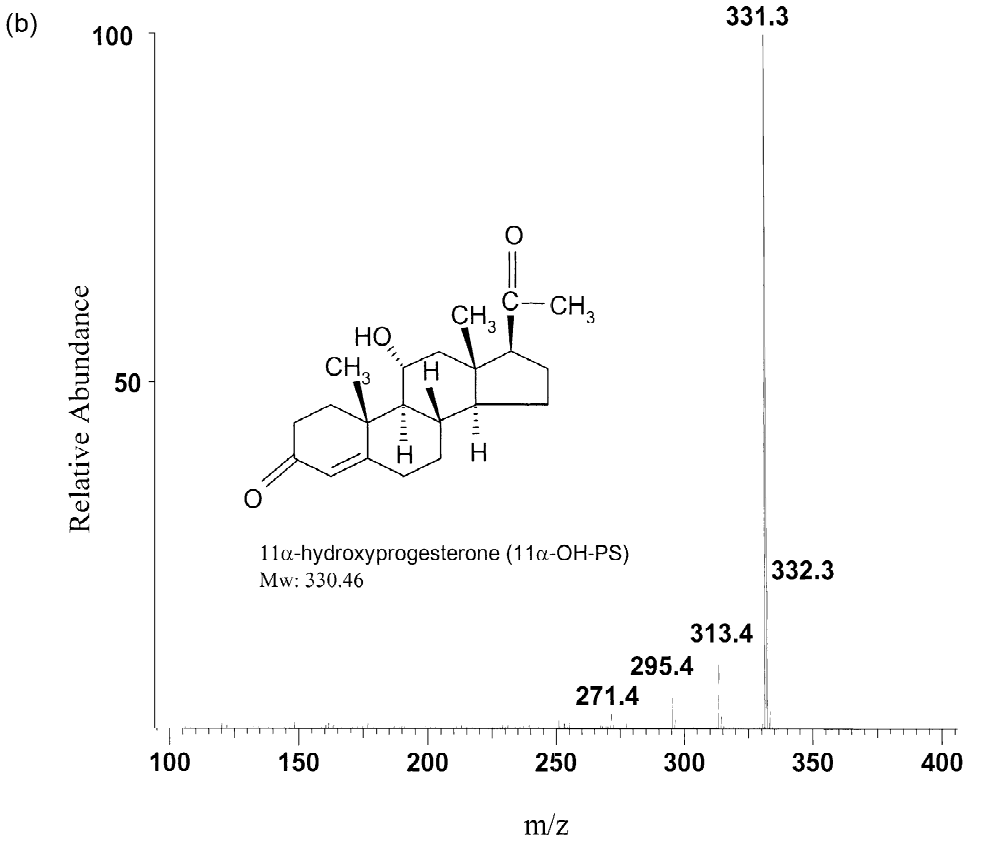


Fig. 4. (*continued*)

hydroxyl group (the product in Fig. 4c) should lose more water from the molecular ion (m/z 331.3) than the hydroxyprogesterone with an equatorial hydroxyl group (Fig. 4b). Thus the m/z peak 313.4 $[M+H-H_2O]^+$ should have a larger intensity for an axial hydroxyl group (9α-OH-PS) than for an equatorial hydroxyl group (11α-OH-PS). As illustrated in the figures, the theory is in line with the results of full scan spectra of the fermentation product, where the relative intensities of the ion at m/z 313.4 is about 50% (Fig. 4c) and for 11α-OH-PS about 10% (Fig. 4b), i.e., the results indicate that the fermentation product obtained probably is an α-OH-PS.

We also investigated the product ion mass spectrum (MS–MS) for 11α-OH-PS, 11β-OH-PS and the fermentation product. The hydroxyl groups of 11β-OH-PS and 9α-OH-PS are axial and that of 11α-OH-PS is equatorial (cf. Fig. 1). The observed product ion mass spectrum for the fermentation product, 9α-OH-PS, with a tertiary axial hydroxyl group is more similar to the spectrum of 11β-OH-PS also with an axial hydroxyl group.

3.3.3. Conclusion of epimer identification

A comparison of the MS–MS spectra above of the fermentation product fragments shows a similar pattern to both that of 11α-OH-PS and 11β-OH-PS, which strongly indicates that the fermentation product is a hydroxyprogesterone. Also the MS^3, MS^4 and MS^5 spectra, respectively of the fermentation product were very similar to corresponding spectra of 11α-OH-PS and 11β-OH-PS. However, without access to a reference substance, it is not possible to decide by mass spectrometric data in which position the hydroxylation of the product has occurred. It might, however, be possible to predict whether the hydroxyl group in the product has an axial or equatorial position. The fermentation product shows

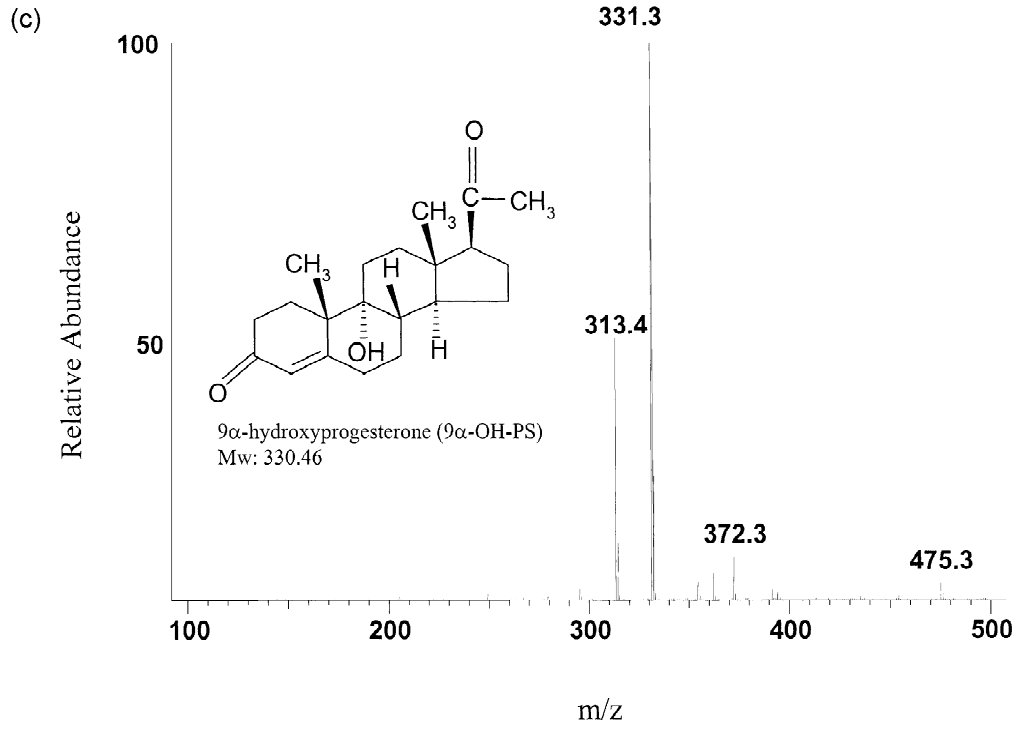


Fig. 4. (*continued*)

a more similar fragmentation pattern to the 11β-OH-PS, with the mono-axial hydroxyl, than to the 11α-OH-PS, with the mono equatorial hydroxyl, which indicates that the fermentation product really has an axial hydroxyl.

3.3.4. High-resolution accurate mass determination

In order to decide the exact molecular mass of the product a high-resolution MS (magnetic sector MS) run was accomplished using two fragments from polyethylene glycol as reference masses. The result shows a molecular mass of 330.2200, to compare with the exact molecular mass for hydroxyprogesterones of 330.21949. All the MS results indicate that the product is a hydroxyprogesterone.

3.4. Preparative LC in two steps for NMR identification

After the experiments with both LC–DAD and MS, it was possible to conclude that the product is a hydroxyprogesterone and probably an axial-hydroxyprogesterone. However, a closer identification was not possible at that stage. Therefore experiments with NMR were undertaken. The NMR runs required around 20 mg of pure product, and hence a preparative LC procedure was undertaken.

3.4.1. Preparative HPLC

Since the analytical Kromasil C_{18} column with 3.5-μm particles showed particularly good characteristics (cf. Table 1) it was logical to use this material also for the preparative separation. However, the semi-preparative column was not available with this particle size. Two semi-preparative columns were used, one reversed-phase C_{18} column and one normal-phase silica column, both of dimensions 250 mm×10 mm I.D. The C_{18} column (Kromasil KR100-5C_{18}) was used with methanol–Milli-Q water (80:20) and the silica column (Kromasil KR100-7-SIL) with the mobile phase ethyl acetate–*n*-hexane (90:10). The performance of the two semi-preparative columns is compared in Table 2 and this shows that there is no drop in performance of the

Table 2
Comparison of retention times (t_R, min), resolution (R_s) and reduced plate height (h) for steroids using two different semi-preparative columns and mobile phase composition

Column/mobile phase	Substance	t_R (min)	R_s	H (μm)	h
Kromasil C_{18} 250×10 (5 μm), MeOH–Milli-Q water (80:20)	11α-OH-PS	8.6		11.8	2.4
	AD	10.1	4.6	10.0	2.0
	11β-OH-PS	11.4	3.6	10.0	2.0
	20α-OH-PS	18.2	14.6	8.9	1.8
	PS	18.9	1.1	8.8	1.8
	20β-OH-PS	25.0	9.3	8.0	1.6
Kromasil Silica 250×10 (7 μm), ethyl acetate–hexane (90:10)	PS	4.2		36.6	5.2
	20β-OH-PS	4.5	2.3	34.5	4.9
	AD	4.7	0.9	29.3	4.2
	20α-OH-PS	5.0	1.3	33.4	4.8
	11β-OH-PS	5.6	4.5	31.7	4.5
	11α-OH-PS	9.2	12.2	35.7	5.1

semi-preparative reversed-phase column compared to the analytical one (cf. Table 1). Note in Table 2 that the reversed elution order when changing from reversed to normal phase was not complete.

3.4.2. Two-step purification

At first, the semi-preparative C_{18} column was used and the extract from the fermentation process was dissolved and separated directly on the reversed-phase system. However, the C_{18} column quickly deteriorated, resulting in over-pressures already at the second and third injections. When the top of the column was visually inspected it was found that a yellow impurity was irreversible adsorbed on the top of the column. The column deterioration occurred although all samples were filtered before the preparative run through a 0.45-μm filter (GHP Acrodisc). In this context, it should be mentioned that the fermentation process liquid had a yellow color; in the case of the rich medium LB the color was strong and in the case of poor M9 medium the color was weak. We used mostly the rich LB medium in the preparative separations.

The yellow impurity was found to be strongly nonpolar; it probably originated from the bacterial cells, which were included when the LB medium was extracted. The LB medium also contains unspecified organic compounds, including lipids, which might interfere with the purification. Because the impurity was strongly nonpolar a better approach might have been to use the preparative silica column. However, on this column the resolution between the product and some impurities was not large enough. Therefore, the product was preparatively purified by a combined use of two principally different separation systems; a normal phase system followed by a reversed-phase system, as described above. This two-step preparative purification was necessary in order to remove the yellow and extremely nonpolar impurity from the process liquid. In the first step, the evaporated extract from liquid–liquid extraction with 6 l fermentation process liquid was re-dissolved and concentrated in 30 ml of the normal-phase mobile phase.

Aliquots of 800 μl of the concentrated extract were injected each time into the semi-preparative silica column (Fig. 5a). Each injection provided fractions from the peak that elutes at 10.1 min (in Fig. 5a), which was the expected product, 9α-OH-PS. The substrate, PS, is the peak eluted at about 7.7 min. Fig. 5a shows that when the extract is from LB the semi-preparative chromatogram contains several substances besides the substrate and the product. However, Fig. 3a,b shows that when the sample has been extracted on a smaller scale, it appears to be less contaminated by other substances. In the second step of the preparative isolation, each normal-phase fraction was evaporated under a stream of N_2 to dryness at 38 °C. Each fraction was later re-dissolved in 500 μl reversed-phase mobile phase and pooled. Thereafter, 800 μl were injected each time into the semi-preparative C_{18} column (Fig. 5b). Each in-

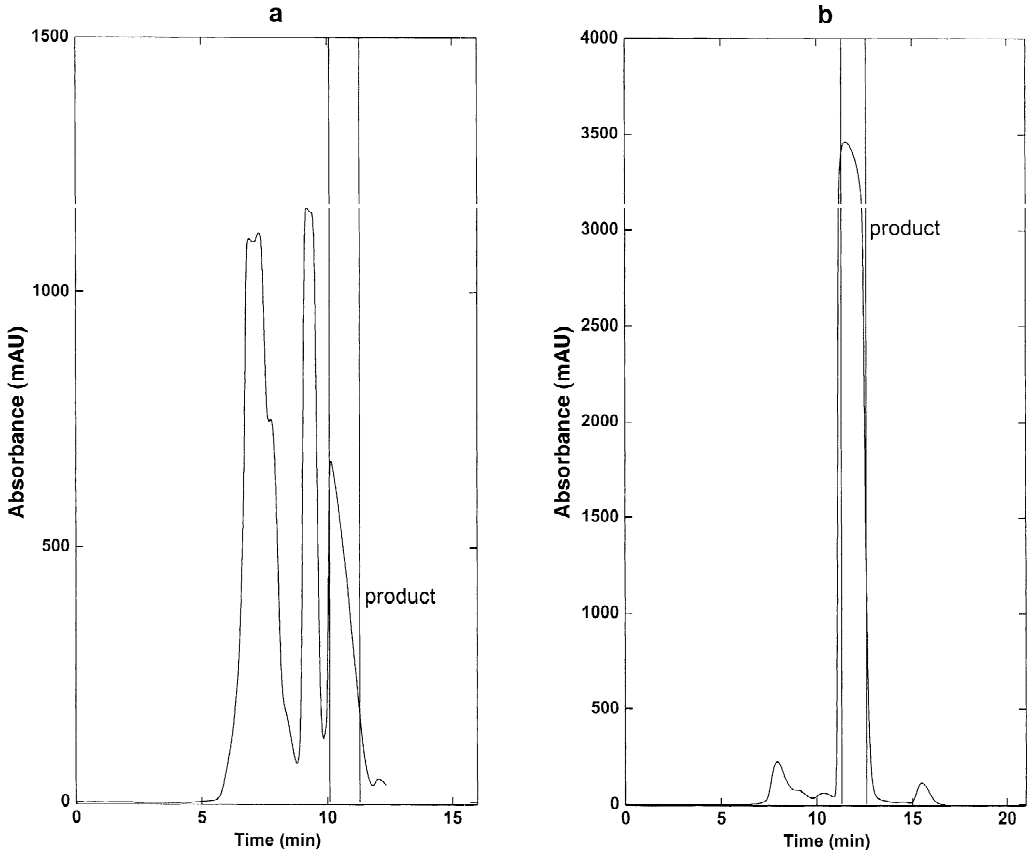


Fig. 5. (a) Record of the first chromatographic purification step. Injection: 800 μl of preconcentrated extract taken from the end of the fermentation process. Chromatographic conditions were HP1100; column: Kromasil KR100-7-SIL (250 mm×10 mm I.D.); mobile phase: ethyl acetate–*n*-hexane (90:10); flow-rate: 2.7 ml/min; UV absorbance detection at 245 nm. Fractions were collected of the peak eluted between 10.1 and 11.3 min (between the lines). (b) Chromatogram obtained from the second purification step. Injection: 800 μl of extract taken from fractions of the first chromatographic purification step. Chromatographic conditions: HP1100; column Kromasil KR100-5-C_{18} (250 mm×10 mm I.D.); mobile phase: methanol–water (80:20); flow-rate: 1.8 ml/min; UV absorbance detection at 245 nm. Fractions were collected of the peak eluted between 11.3 and 12.6 min (between the lines).

jection into this system yielded a peak that elutes at 11.6 min (Fig. 5b), which contained the expected product. These fractions were evaporated to dryness under a stream of N_2 at 38 °C to give a pure crystalline material. This material was analyzed using two different mobile phases with the column Kromasil KR100-3.5C_{18} (150 mm×4.6 mm I.D.); first with a mobile phase composed of methanol–HPLC-grade water (70:30), and a second time with acetonitrile–HPLC-grade water (55:45). Only one peak appeared in either phase system and the peak purity test showed that the peak was pure.

3.5. NMR identification

The isolated compound was identified by comparison of the ^{13}C NMR spectrum with reported data for 9α-hydroxyprogesterone [25–28]. Chemical shifts for all carbon atoms were identical within 0.1 ppm, and considerably different from those for 9β-hydroxyprogesterone (Table 3). Since no authentic reference material was available to us, we also carried out a complete NMR signal assignment (^1H and ^{13}C) using standard NMR methodology (see Experimental). Furthermore, the stereochemistry of

Table 3
^{13}C NMR spectra data for the product and of literature data for 9α-OH-PS and 9β-OH-PS

Index	Observed	Assignment	Lit. 9α	Assignment	Lit. 9β
1	209.1	20	209.2	20	209.0
2	198.9	3	198.9	3	199.1
3	168.1	5	168.1	5	170.1
4	127.1	4	127.1	4	125.7
5	76.3	9	76.4	9	76.5
6	63.2	17	63.3	17	63.7
7	49.4	14	49.5	14	50.1
8	44.4	10	44.5	10	44.0
9	43.7	13	43.7	13	44.8
10	37.3	8	37.4	8	44.5
11	34.1	12	34.2	12	37.7
12	34.0	2	34.0	2	33.8
13	31.7	6	31.7	6	29.5
14	31.4	21	31.5	21	31.3
15	28.5	1	28.5	1	29.5
16	26.8	11	26.8	11	32.5
17	25.3	7	25.4	7	23.9
18	24.2	15	24.2	15	23.1
19	22.8	16	22.9	16	25.0
20	19.9	19	19.9	19	23.3
21	12.4	18	12.5	18	12.9

Index = Lines sorted by chemical shift. Assignment was derived from further experiments. Experimental conditions: ^{13}C NMR spectra were recorded for C^2HCl_3 solutions at 100.6 MHz at 25 °C, chemicals shifts are reported in ppm referenced to tetramethylsilane.

the compound, i.e., the α-position of the hydroxyl group, was confirmed by showing that H-8 and H-11β are on the same face of the molecule as Me-19. This was done by detection of nuclear Overhauser effects (NOEs) between these protons (arrows in Fig. 6).

4. Conclusions

The usefulness of LC–DAD and MS for rapid and preliminary characterization of the formed product was demonstrated during a typical exploratory scale biotechnological synthesis of a component whose

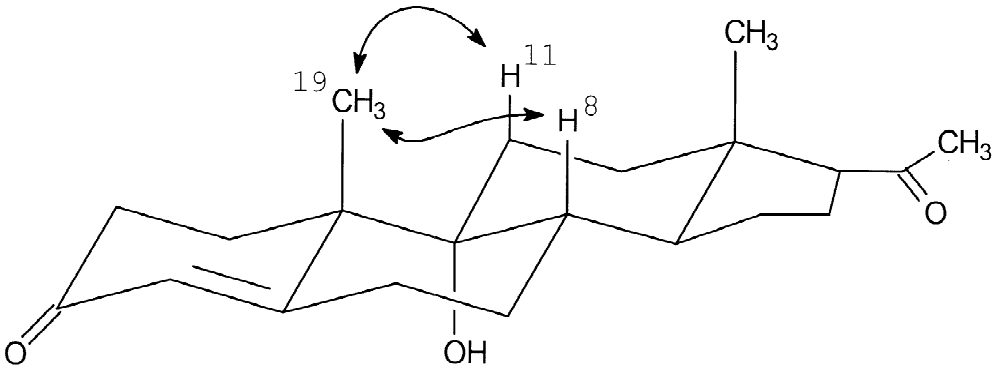


Fig. 6. Stereochemically important nuclear Overhauser effects (arrows) observed for 9α-OH-PS.

verification was hampered by the lack of commercially available reference material. Here the biotechnological conversion of progesterone to 9α-hydroxyprogesterone was chosen as model system. The gene for steroid 9α-hydroxylase was identified, processed, and cloned into *E. coli* to express a protein with activity towards progesterone.

It was found that the product could rapidly be preliminarily identified by a combined analysis of its retention time and its UV spectrum, registered on-the-fly, by DAD. The great advantage of LC–DAD is that it is rapid and capable of directly telling whether a product with the expected spectrum is produced. If this is not the case, then the synthesis can be stopped immediately without a complete characterization by other spectroscopic techniques, thus saving time and money.

ESI-MS provided us with the exact molecular mass of the product and confirmed that the product is a hydroxylated progesterone. This is in general important information, but not in the context of deciding whether to stop or continue the biotechnological synthesis considering that the combination of the chromatographic and spectroscopic information provided by the LC–DAD already strongly indicated that a hydroxylation of the substrate had occurred. Mass spectrometry could unfortunately neither tell us the position nor the orientation of the hydroxyl group on the steroid skeleton. However, NMR spectroscopy provided the definite proof of the product identity, 9α-hydroxyprogesterone. While LC–DAD and MS are sensitive techniques giving signals with small amounts of analytes (nano- to femtomoles), NMR requires much larger amounts to give adequate signals, which means that time-consuming preparative isolations of the analyte/product have to be done before measurement. The technique is consequently not suitable for rapid on-line analysis in process control.

Acknowledgements

The authors thank Amersham Biosciences and The Swedish National Board for Industrial and Technical Development (NUTEK) for financial support of this project. We are grateful to Rickard Johannisson for doing the fermentation experiments and to Adolf Gogoll for NMR experiments and evaluation.

References

[1] S.D. Sarker, R. Lafont, J.P. Girault, V. Sik, L. Dinan, Pharm. Biol. 36 (1998) 202.
[2] P. Sievert, W.S. Hancock (Eds.), New Methods in Peptide Mapping for the Characterization of Proteins, CRC Press, New York, 1996.
[3] N. Furusawa, Biomed. Chromatogr. 15 (2001) 235.
[4] R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry—Fundamentals, Instrumentation and Applications, Wiley, New York, 1997.
[5] C. Hao, R.E. March, Int. J. Mass Spectrom. 212 (2001) 337.
[6] K. Shimada, K. Mitamura, T. Higashi, J. Chromatogr. A 935 (2001) 141.
[7] W.J. Marsheck, US Pat. 4 397 947 (1983).
[8] K. Kieslich, US Pat. 4 237 220 (1980).
[9] R Arnell, R. Johannisson, J. Lindholm, A. Ballagi, B. Ersson, K. Caldwell, in preparation.
[10] L. Mueller, J. Magn. Reson. 72 (1987) 191.
[11] G. Bodenhausen, D.J. Ruben, Chem. Phys. Lett. 69 (1980) 185.
[12] K.E. Kövér, O. Prakash, V.J. Hruby, Magn. Reson. Chem. 31 (1993) 231.
[13] D.J. States, R.A. Haberkorn, D.J. Ruben, J. Magn. Reson. 48 (1982) 286.
[14] A.A. Bothner-By, R.L. Stephens, J.M. Lee, C.D. Warren, R.W. Jeanloz, J. Am. Chem. Soc. 106 (1984) 811.
[15] J.K.M. Sanders, J.D. Mersh, in: J.W. Emsley, J. Feeney, L.H. Sutcliffe (Eds.), Prog. Nucl. Magn. Reson. Spectrosc., Vol. 15, Pergamon Press, Oxford, 1982, p. 353.
[16] T. Parella, Magn. Reson. Chem. 36 (1998) 467.
[17] R. Cavalli, E. Peira, O. Caputo, R. Gasco, Int. J. Pharm. 182 (1999) 59.
[18] S. Lin, N. Kohyama, H. Tsuruta, Ind. Health 34 (1996) 143.
[19] B. Agnus, N. Gosselet, B. Sebille, J. Chromatogr. A 663 (1994) 27.
[20] D.N. Kirk, S.J. Gaskell, B.A. Marples, in: H.L.J. Makin, D.B. Gower, D.N. Kirk (Eds.), Steroid Analysis, Blackie, Glasgow, 1995, p. 25.
[21] J. Lindholm, M. Johansson, T. Fornstedt, J. Chromatogr. B, submitted.
[22] T.B. Vree, C.P.W.G.M. Verwey-van Wissen, A.J. Lagerwerf, A. Swolfs, R.A.A. Maes, R.D. van Ooijer, O.R. van Eikema Hommes, P.J.H. Jongen, J. Chromatogr. B 726 (1999) 157.
[23] K.-E. Karlsson, personal communication.
[24] V.I. Zaretskii, N.S. Wulfson, V.G. Zaikin, L.M. Kogan, N.E. Voishvillo, I.V. Torgov, Tetrahedron 22 (1996) 1399.
[25] J.F. Tempelton, V.P. Sashi Kumar, K. Marat, R.S. Kim, F.S. Labella, D. Cote, J. Nat. Prod. 50 (1987) 463.
[26] S.H. Hu, G. Genain, R. Azerad, Steroids 60 (1995) 337.
[27] J. Favero, T. Ton That, F. Winternitz, Bull. Soc. Chim. Fr. 1–2 (1979) 56.
[28] D.N. Kirk, H.C. Toms, C. Douglas, K.A. White, K.E. Smith, S. Latif, R.W.P. Hubbard, J. Chem. Soc., Perkin Trans. 2 (1990) 1567.