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Microbial Models of Drug Metabolism: Microbial Transformations of Trimegestone® (RU27987), a 3-Keto-$\Delta^{4,9(10)}$-19-norsteroid Drug

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Abstract—Screening microorganisms for the biotransformation of the 3-keto-$\Delta^{4,9(10)}$-19-norsteroid **RU27987** (Trimegestone®) resulted in the isolation of nine identified metabolites, some of them being selectively produced by different strains. Eight metabolites were found to be hydroxylated on various positions of the rings, and one was additionally epoxidized. These microbial metabolites could be used as chromatographic standards and two of them were found identical to the unknown major human metabolites. Moreover, most microbial metabolites were produced in sufficient amounts to be tested for their biological activities. All these features demonstrate the usefulness and versatility of microbial biotransformation systems as a tool for early identification and convenient production of potentially active mammalian and non-mammalian metabolites.

Introduction

Since the last 40 years, microbial transformations for the production of steroid drugs have received a widespread attention.[1–8] The ability of filamentous fungi to perform regio- and stereoselective hydroxylation of common steroidal compounds is now a well known and industrially exploited bioconversion method. On the other hand, steroid hydroxylation is of physiological importance in mammalian organisms due to detoxication of exogenous steroid drugs. Thus, the limited microbial transformation of such drugs can be an efficient source of useful information about their metabolism in mammals, in connection with the now well-accepted concept of 'microbial models of mammalian metabolism'.[9–19] In addition, it can be a convenient way to produce in sizable amounts new steroid drug metabolites otherwise difficult or impossible to synthesize by chemical methods. This report describes the microbial biotransformation of **RU27987** (Trimegestone®, **1**), a 3-keto-$\Delta^{4,9(10)}$-19-norsteroid drug which has been recently developed by Hoechst–Marion–Roussel as a progestomimetic and a regulator of calcium assimilation for the therapy of osteoporosis. This drug appeared as an interesting example to investigate by such method: being very active, it had to be used at very low doses in experiments with animals and women, and the amount of recovered main metabolites was not sufficient to allow their unambiguous structural identification. Moreover, only very few microbial transformations of such Δ-$^{4,9(10)}$ steroids have been described up to now.[20]

Results

Preliminary screening

A limited screening of 41 fungal strains and 10 *Streptomyces* strains currently used for steroid hydroxylation reactions[2–6,21] was undertaken, using 65-h grown fungal cultures to which was added a concentrated ethanol solution of **RU27987** (**1**, 0.5 g L^{-1} final concentration), and incubated with stirring at 27°C. Most of the strains tested were able to metabolize extensively this substrate within a 1- to 5-day period (Table 1), producing in the incubation medium variable amounts of at least nine metabolites, completely separated by HPLC (see Fig. 1)

Key words: Fungi; oxygenation; stereospecificity; steroids.

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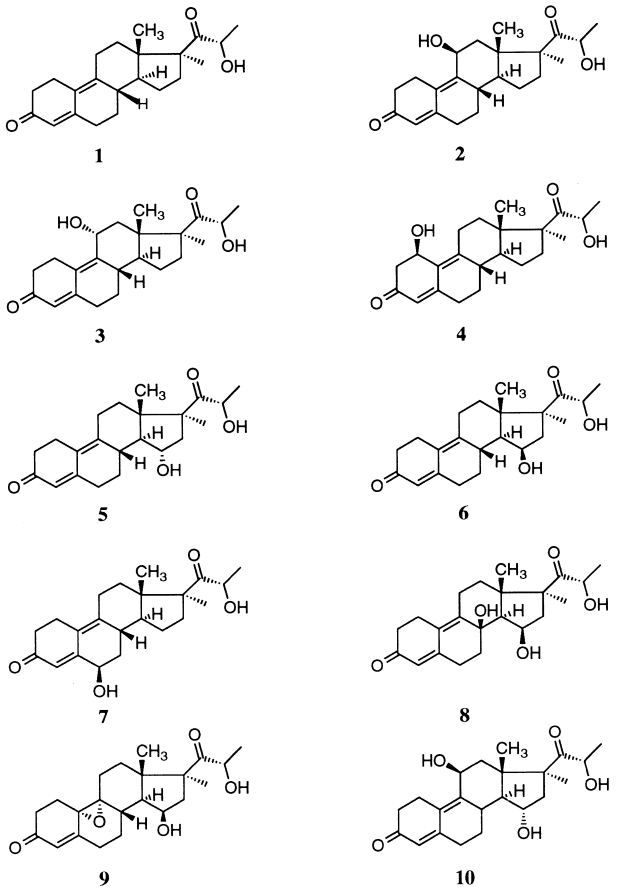

and easily detected by their characteristic UV-absorbance. These metabolites were named **2** to **10** and characterized by their elution times from the reverse-phase column. While several of them were uniformly present, sometimes in large amounts, such as **2**, some of them, noticeably, were only produced by one or a few strains, sometimes as a major or one of the major metabolites, thus facilitating the selection of the best strain to be used for the preparation of each one of those metabolites. Only metabolite **8** was poorly represented in all strains, which necessitated its isolation as a minor product from an incubation with *A. corymbifera*. Concerning compound **9**, it was formed only after prolonged incubation times with *C. echinulata* var. *echinulata* (= *C. bainieri*) (Fig. 2), indicating a possible secondary reaction occurring on one of the initial metabolism products.

Yield and time course of RU27987 metabolism

Taking advantage of the characteristic UV-absorbance maximum at $\lambda = 310$ nm for the substrate and related metabolites retaining the conjugated ketodiene chromophore, and using analytical HPLC separations, it was possible to evaluate directly from an aliquot of the incubation filtrate the time course of **RU27987** biotransformations and the production of most metabolites, using an external standardization method with **RU27987** or metabolite **2** as standards. The kinetics of product formation is shown, for the most significant strains, in Fig. 2. With such strains, while **RU27987** disappeared within a few hours from the incubation supernatant, probably by physical adsorption on the biomass, the total recovery of the main products, measured from the supernatant after a 24- to 48-h incubation period, generally accounted for 80–85% of the substrate added, indicating an effective and quantitative transformation, associated with a major location of the metabolism products outside of the mycelial cells. When secondary oxidation occurred, the filiation of metabolites could be easily deduced from the kinetic monitoring of the main metabolites formed (Fig. 2).

Identification of RU27987 transformation products

All metabolites were isolated and purified from moderate-scale transformations (using 0.25–0.5 g L^{-1} of **RU27987**) with selected strains, incubated at 27°C during optimized periods (see Fig. 2). After solvent extraction from the incubation filtrates, the metabolites were separated by silica gel flash chromatography, followed by further purification by crystallization or preparative HPLC on a reverse-phase column.

The structure elucidation of metabolites **2** to **10** relied primarily on mass spectrometric measurements, which indicated the number of oxygen atoms introduced into the substrate molecule, then on the examination of the changes in the ^1H (Table 2) and ^{13}C NMR spectra (Table 3) and their interpretation, using two-dimensional homo- and heteronuclear correlation experiments to perform a complete assignment of all hydrogen and carbon resonances. The multiplicity assignment of carbon atoms by a DEPT technique was used to identify and differentiate hydroxylation either on a secondary or a tertiary carbon atom. A major difficulty encountered with hydroxylated derivatives of steroids (i.e. the precise location of the newly introduced hydroxyl group(s)) was generally solved by the following elements: the coupling constants, in ^1H NMR spectra, of the new mid-field -C*H*OH-signal (when existing) or its half-height width characterized an axial or equatorial position; the eventual occurrence of a significant downfield shift of the 18-methyl group signal (and/or the methyl group borne by C-17), indicated a characteristic 1,3-*cis*-diaxial interaction with a hydroxyl group. In addition, the coupling characteristics of most hydrogen atoms, observed in ^1H-^1H COSY and TOCSY experiments, were used to assign the signals of vicinal or proximate protons. HMQC and HMBC experiments were also used to relate individual hydrogens to vicinal or proximate carbon atoms. NOESY experiments were used to confirm the spatial proximity of hydrogen atoms. Final structural assignments resulted from a consistent interpretation of all spectral data. However, modelling studies of **RU27987** and hydroxylated metabolites by energy minimization revealed relatively flexible conformations[22–24] for the A-ring (C-1 and C-2), and the B-ring (C-6 and C-7), which had to be accounted for in the calculation of dihedral angles in order to be related to hydrogen coupling (^3J) constants. At last, most metabolites were found to retain a UV-absorbance maximum at about 310 nm, corresponding to the initial diene-conjugated carbonyl chromophore; any shift of

Table 1. Screening for the metabolism of **RU27987** (**1**) by selected microbial strains (filamentous fungi and *Streptomyces*)[a]

Microorganisms	Metabolites								
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Absidia corymbifera LCP 63.1800	+	−	−	−	+ + +	−	+ +	−	−
Absidia cylindrospora LCP 57.1569	+	+	±	−	+ + +	−	−	−	−
Acremonium alternatum MMP 3010	+ + +	+	+	±	+	−	−	−	−
Aspergillus alliaceus NRRL 315	±	±	−	±	+ + +	−	−	−	−
Aspergillus candidus ATCC 20023	±	−	−	−	±	−	−	−	−
Aspergillus terreus LCP 75.2296	+ + +	±	+ +	+	+ + +	+ +	−	−	−
Circinella minor MMP 1837	+ +	+ + +	±	−	+ + +	−	−	−	−
Cunninghamella echinulata var.*echinulata* ATCC 9244	+ +	+	+ + +	−	+ +	+	±	(+ +)[b]	−
Cunninghamella echinulata NRLL 3655	+ +	±	+	−	±	−	−	−	−
Cunninghamella echinulata LCP 73.2203	+ +	±	+	−	+	−	−	−	−
Cunninghamella elegans ATCC 26269	+ + +	+	+ +	±	+ +	±	+	−	−
Cunninghamella elegans ATCC 36112	+ + +	+	+	±	±	±	±	−	−
Curvularia lunata NRRL 2380	+ + +	−	−	−	−	−	−	−	−
Fusarium roseum ATCC 14717	−	±	±	+ + +	−	+	−	−	−
Mortierella isabellina NRRL 1757	+ + +	+ +	+ +	±	+	+ +	−	−	−
Mortierella isabellina LCP 52.108	+ +	+	+ + +	±	±	+	−	−	−
Mucor circinelloides CBS 108-16	+ +	±	−	−	±	−	−	−	−
Mucor griseocyanus ATCC 1207a	+ + +	−	−	−	−	−	−	−	−
Mucor hiemalis BO	+ + +	−	−	−	−	−	−	−	−
Mucor plumbeus CBS 110-16	+ +	−	−	−	−	−	−	−	−
Mucor plumbeus ATCC 4740	+ +	−	−	−	−	−	−	−	−
Mucor racemosus BO	+ + +	+	−	−	−	−	−	−	−
Mucor rouxii CBS 416-77	+ +	±	−	−	±	−	−	−	−
Rhizopus arrhizus ATCC 11145[c]	+ + +	+	+ +	+ +	+	+ +	±	±	−
Streptomyces fradiae NRLL B1195	+ +	−	−	−	−	−	−	−	−
Streptomyces platensis NRRL 2364	+ +	−	−	−	−	−	−	−	−
Streptomyces rimosus NRRL 2234	+ +	−	−	−	−	−	−	−	+
Syncephalastrum racemosum LCP 72.2150	+ +	+	+	−	+ + +	−	−	−	−
Thamnostylum piriforme ATCC 8992	+ + +	±	±	−	±	±	−	−	−

[a] All incubations were performed at 0.5 g/L during 24–48 h at 27°C. Metabolites observed (detected by HPLC) are quoted as follows: −, absent; ±, weak formation (< 5% of the total); +, 5–15%; + +, 15–30%; + + +, major product (> 30%).
[b] Metabolite appearing only on prolonged incubation times (> 72 h).
[c] The incubation filtrate contained several minor unidentified products.

this absorbance to lower wavelengths was interpreted as a modification of one of the conjugated double bonds. However, all of the metabolites have at least retained the 3-keto-4-ene structure, as deduced from the characteristic H-4 NMR signal (sharp singlet at 5.7–6 ppm), and the unchanged methyl substituted α-ketol side-chain, as shown by distinctive ^{1}H and ^{13}C NMR signals (see Tables 2 and 3).

The most common metabolite **2**, which was formed as a nearly exclusive product by several strains (see Table 1 and Fig. 1), was obtained from a 48-h incubation with *M. hiemalis* BO (80% yield as determined by HPLC, 55% isolated yield). From mass spectrometry data, this metabolite (MH^+ at m/z 359) appeared to be a monohydroxylated derivative of **RU27987**. Axial hydroxylation on a -CH_2- group having one or two vicinal hydrogen atoms was indicated in the ^{1}H NMR spectrum by the presence of a broad one-proton doublet at 5.08 ppm ($J = 3.5$ Hz), whereas this high chemical shift suggested an allylic position such as C-1, C-6, or C-11, or a C-2 position adjacent to the carbonyl group. All signals of the side chain or the methyl group on C-17 were nearly unchanged, but a strong downfield shift of 18-CH_3 ($\Delta\delta = +0.2$ ppm) indicated a 1,3-*cis*-diaxial interaction with the newly introduced hydroxyl group, characterizing **2** as an 11β-hydroxy derivative of **RU27987**, as confirmed by comparison with an authentic synthetic sample.

Metabolite **3** was apparently formed together with **2** by most strains, generally in much lower amounts. However, *A. cylindrospora* LCP 57.1569 formed nearly equal amounts of both metabolites (see Fig. 1), while *C. minor* MMP 1197 was the only strain to produce a larger amount of **3** (about 20% by HPLC, see Fig. 1). A slow conversion of **3** to **2** seemed to occur in the incubation conditions; this conversion was greatly accelerated by heating the incubation filtrate before extraction, suggesting an isomerisation of an 11α-allylic alcohol group to a favored 11β-hydroxylated position. This was entirely confirmed by ^{1}H NMR data of **3**, which were very similar to those of the 11β-derivative, except for H-11, found as a doublet of triplets at 4.76 ppm (versus 5.08 ppm in the 11β-OH derivative) and a 18-CH_3 signal at 0.8 ppm (versus 1 ppm). All assignments could be further ascertained by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. Noticeably, a particularly significant low field value (3.45 ppm) was found for the ^{1}H chemical shift of one of the 1-hydrogens, due to the deshielding effect of the (*eq*)-11α-hydroxyl group.[25]

Metabolite **4**, another monohydroxylated product of **RU27987**, was formed as a major metabolite by *C.*

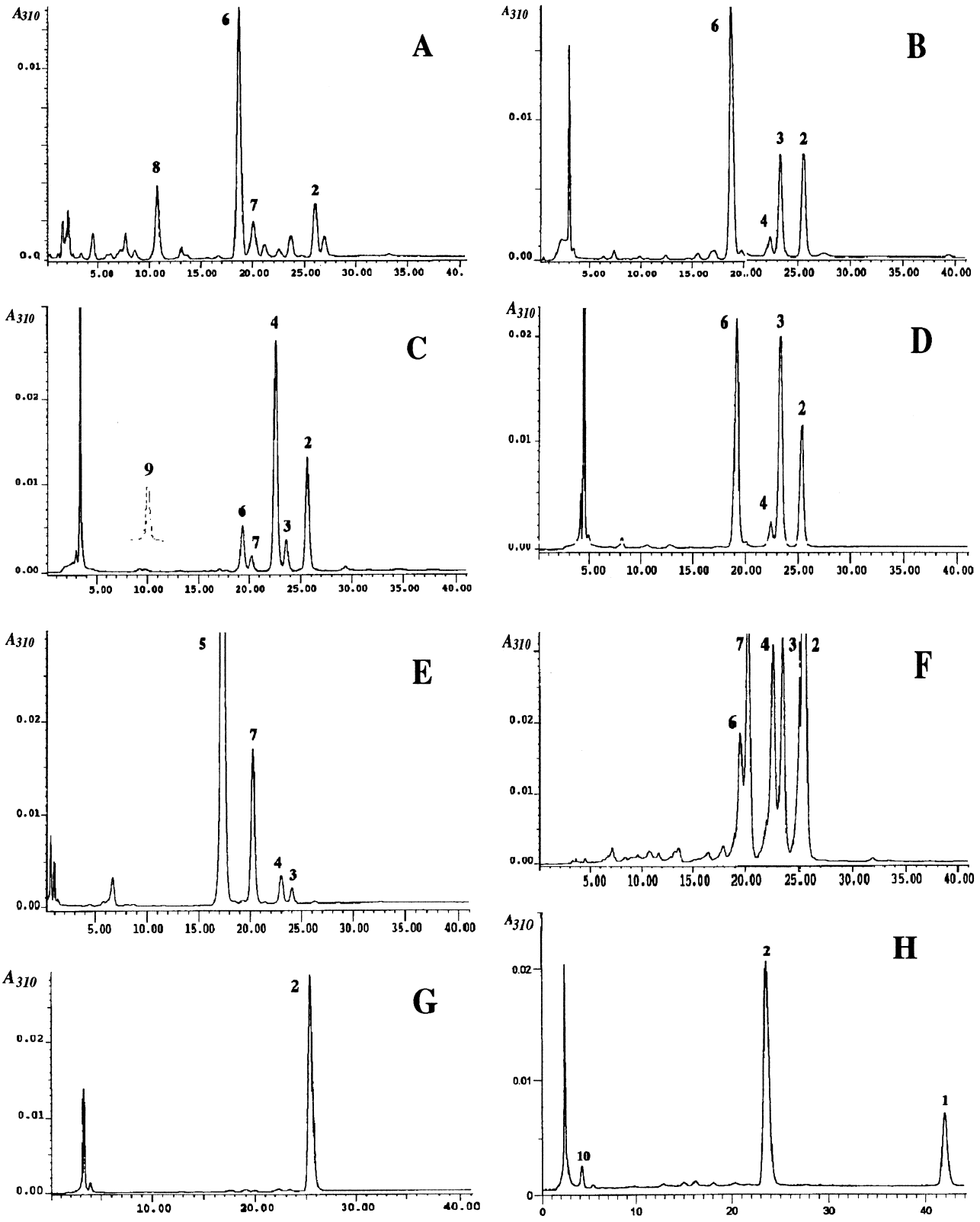

Figure 1. HPLC profiles of 48 h-incubation supernatants of **RU27987** (0.5 g/L) with *A. corymbifera* (A), *A. cylindrospora* (B), *C. echinulata* var. *echinulata* (C), *C. minor* (D), *F. roseum* (E), *M. isabellina* (F), *M. hiemalis* (G), *S. rimosus* (H). Detection at 310 nm, expected for metabolite **9**, detected at 244 nm.

echinulata ATCC 9244 (about 25% yield by HPLC, 11% isolated yield). Very little changes observed in ^{1}H and ^{13}C NMR signals of 18-CH_3 and of side chain and methyl group borne by C-17 excluded any hydroxylation in most positions of the CD rings. A ^{1}H NMR signal at 5.11 ppm (dd, $J=3$ and 4.5 Hz) indicated a low-field new -C*H*OH- group, apparently coupled with only two protons, and an HMBC experiment

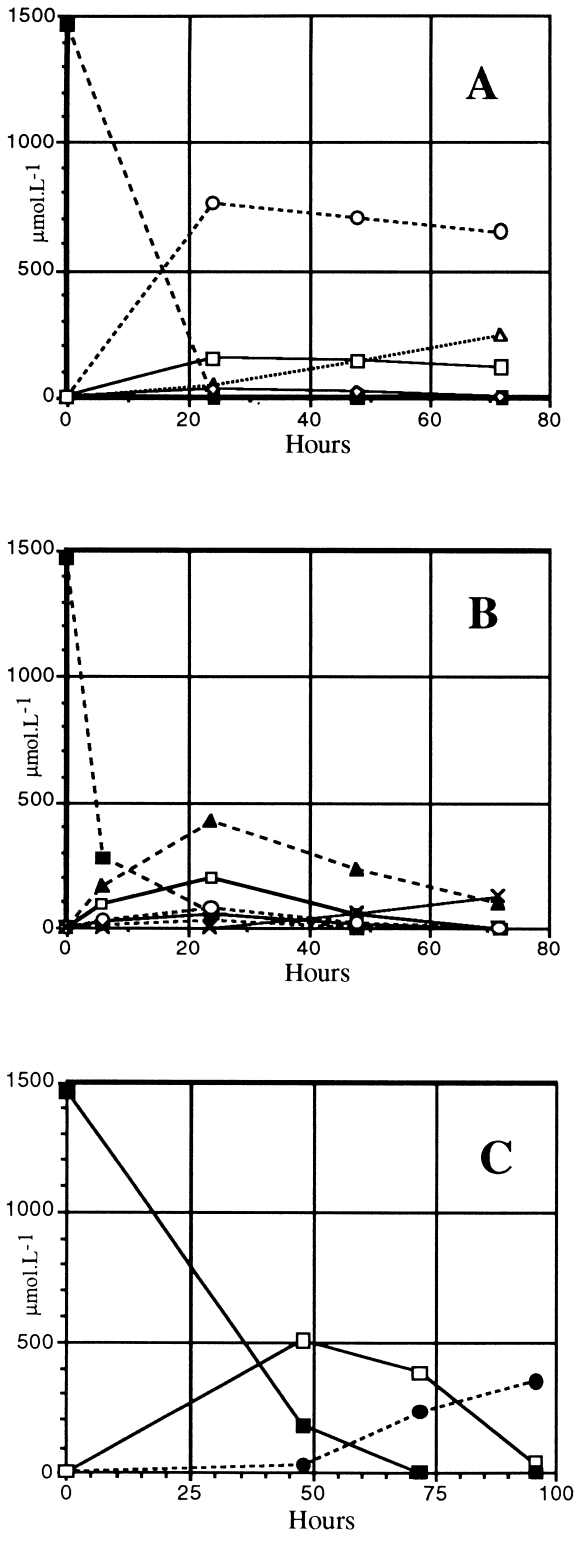

Figure 2. Time course of the biotransformation of Trimegestone (0.5 g/L) by *A. corymbifera* (A), *C. echinulata* var. *echinulata* (B) and *S. rimosus* (C). ---■--: residual Trimegestone; —□—: metabolite **2**; —◇—: metabolite **3**; ---▲---: metabolite **4**; ----○----: metabolite **6**; ----▼----: metabolite **7**; ----△----: metabolite **8**; —X—: metabolite **9**; ----●----: metabolite **10**.

revealed a coupling with C-3, suggesting hydroxylation in positions-1 or -2. The high deshielding of the equatorial H-11α (at 3.03 ppm), symmetrical to that found for the 11α-hydroxylated derivative **3** (see above), suggested a location in position-1. All other assignments were in agreement with the structure of a 1-hydroxy derivative for metabolite **4**. The absolute stereochemistry of the OH group was otherwise difficult to determine with certainty. A pseudoaxial position of the hydroxyl group was inferred from the relatively small coupling constants ($J = 3$ and 4.5 Hz) measured for the C*H*OH hydrogen. However, owing to the flexibility of the A ring, α- or β-axial configurations were equally possible; a modelling of both isomers (Fig. 3) showed that the lower energy conformation should correspond in each case to an axial OH group.

The weak shifting of the 8β-hydrogen ($\Delta\delta = +0.06$ ppm) and the deshielding of the 14α-hydrogen ($\Delta\delta = +0.21$ ppm), similar to the features previously observed for the 11α-derivative **3** (see Table 2), and opposed to those observed with the 11β-derivative **2**, would suggest a 1α-position for the hydroxyl group. However, NOESY experiments showed a significant enhancement only for the 11α-H (at 3.03 ppm), upon irradiation of the 1-CH(OH) hydrogen (at 5.11 ppm). Measurement of hydrogen distances in the minimized models[24] of 1α- and 1β-hydroxy derivatives (with a 1-OH group axially disposed) pointed up a higher difference between the distances of 11α/11β protons to the C-1 hydrogen in the 1β-hydroxy derivative (2.1/3.2 A), compared to the 1α-hydroxy derivative (2.2/2.5 A), thus explaining the NOE-detection of only one (H-11α) of the C-11 hydrogens.

Crystallisation of an acetate derivative was attempted, but it turned up that the acetylation product was a phenolic compound **11**, resulting from acetate elimination and rearrangement as shown in Figure 4. At last, crystallisation of pure metabolite **4** from a saturated solution in CH_2Cl_2-acetone afforded nice crystals, allowing an unambiguous X-ray determination (Fig. 5) and definitely establishing a 1β-hydroxy configuration.

Metabolite **5** was exclusively formed by two strains, *F. roseum* ATCC 14717 and *R. arrhizus* ATCC 11145. The latter produced a number of other metabolites, while the former produced **5** as a major metabolite (65% by HPLC), thus facilitating its purification (45% isolated yield). Metabolite **5** was a monohydroxylated derivative of **RU27987** (MH^+ at m/z 359), resulting from the hydroxylation of a -CH_2- group, as shown by ^{13}C NMR spectra. No change in the ^1H NMR chemical shifts of 18-CH_3 and the side-chain signals was observed, while the signal of the methyl group borne by C-17 was significantly downfield shifted ($\Delta\delta = +0.15$ ppm), suggesting hydroxylation in position-15α or -16. Careful examination of the ^1H-^1H COSY correlation spectra revealed a coupling of the new -C*H*(OH)- group with three protons, including a pseudo-diaxial coupling with H-14 ($J \geq 9$ Hz), and establishing the structure of **5** as that of a 15α-hydroxy derivative of **RU27987**. A complete and consistent assignment of all hydrogen and carbon atoms followed, using HMBC and HMQC experiments.

Metabolite **6** was formed as a major product in several strains (e.g. *A. corymbifera* LCB 63.1800) *A. cylindrospora*

Table 2. Complete assignments of ^{1}H NMR spectra of **RU27987** and metabolites **2**–**10** (δ ppm, *J* Hz; 500.13 MHz); solvent: **2**–**9**, $CDCl_3$; **10**, MeOH

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
H-1α	2.50 (m)	2.74 (m)	2.89 (m)	5.11 (dd) *J* = 3, 4.5	2.50 (m)	2.57 (m)	2.63 (m)	2.60 (m)	2.05 (m)	2.76 (m)
H-1β	2.89 (m)	2.95 (dt) *J* = 5.4, 15.3	3.45 (dt) *J* = 7.3, 16.1	—	2.85 (m)	2.92 (dt) *J* = 4.5, 14.4	2.91 (dt) *J* = 5.6, 15.4	2.92 (m)	2.35 (dd) *J* = 4.8, 13.2	2.96 (dt) *J* = 5.9, 14
H-2α	2.45 (dd) *J* = 5, 11.5	2.48 (m)	2.45 (m)	2.64 (dd) *J* = 4.2, 16.9	2.50 (m)	2.48 (m)	2.50 (dt) *J* = 5.6, 12.6	2.47 (m)	2.55 (dt) *J* = 4, 16.8	2.46 (m)
H-2β				2.74 (dd) *J* = 3, 16.9					2.78 (m)	
H-4	5.67 (s)	5.77 (s)	5.79 (s)	5.79 (s)	5.67 (s)	5.71 (s)	5.84 (s)	5.78 (s)	6.06 (s)	5.76 (s)
H-6α	2.34 (dt) *J* = 2.4,13.8	2.47 (m)	2.41 (m)	2.42 (m)	2.38 (m)	2.45 (m)	4.40 (dd) *J* = 2.8, 5	2.30 (m)	2.39 (m)	2.42 (m)
H-6β	2.40 (dt) *J* = 3.6,16		2.51 (dt) *J* = 3.6, 15.3	2.56 (ddd) *J* = 3.4, 5.9, 16	2.48 (m)		—	2.89 (m)		2.56 (dt) *J* = 4.2, 15
H-7α	1.29 (m)	1.33 (m)	1.33 (m)	1.45 (m)	1.60 (dt) *J* = 3.2, 14	1.40 (m)	1.52 (ddd) *J* = 2.8, 9, 13.7	1.72 (dd) *J* = 3.7, 13.9	1.64 (m)	1.68 (m)
H-7β	1.89 (m)	1.96 (m)	1.91 (m)	1.87 (m)	2.14 (m)	2.18 (m)	2.06 (dt) *J* = 5, 13.7	2.23 (dt) *J* = 3.4, 13.9	1.92 (m)	2.18 (m)
H-8β	2.23 (br.t) *J* = 11	2.57 (m)	2.18 (m)	2.29 (m)	2.47 (m)	2.70 (m)	2.56 (m)	—	2.60 (ddd) *J* = 3, 5, 12.6	2.8 (m)
H-11α	2.14 (dt) *J* = 4.3,13.6	5.08 (br.d) *J* = 3.7	—	3.03 (ddd) *J* = 2, 5, 15	2.14 (m)	2.20 (m)	2.22 (m)	2.62 (m)	1.74 (dt) *J* = 3.7, 14.3	5.05 (br.d) *J* = 3.7
H-11β	2.81 (br.d) *J* = 16	—	4.76 (dt) *J* = 6, 9	2.37 (dt) *J* = 5.9, 15	2.85 (m)	2.86 (m)	2.84 (ddd) *J* = 2.1, 4.9, 16.1	2.78 (dt) *J* = 3.9, 15.2	2.10 (dd) *J* = 3.9, 14.3	—
H-12α	1.94 (ddd) *J* = 2, 5.2, 12.2	1.91 (dd) *J* = 4.8, 14.1	1.91 (dd) *J* = 9, 12	1.74 (dt) *J* = 5, 13	1.80 (dd) *J* = 4.4, 12.7	1.69 (dt) *J* = 4.4, 12.2	1.70 (dt) *J* = 5, 12.6	1.68 (m)	1.64 (m)	1.95 (dd) *J* = 4.7, 14.1
H-12β	1.68 (dt) *J* = 4.6, 13	2.22 (dd) *J* = 1.7, 14.1	2.29 (dd) *J* = 6, 12	2.05 (ddd) *J* = 2, 5.5, 12.5	1.92 (ddd) *J* = 2, 5.2, 12.3	1.90 (m)	2.00 (m)	1,95 (dt) *J* = 3.9, 11.6	1.88 (m)	2.18 (m)
H-14α	1.59 (dt) *J* = 7.6,11.8	1.59 (m)	1.81 (m)	1.80 (m)	1.71 (dd) *J* = 8.8, 12	1.54 (dd) *J* = 5.7, 12.2	1.68 (m)	1.63 (d) *J* = 5.6	1.91 (m)	1.68 (m)
H-15α	1.32 (m)	1.40 (m)	1.32 (m)	1.40 (m)	—	4.35 (dt) *J* = 2, 6.6	1.38 (m)	4.66 (t) *J* = 5.7	4.30 (dt) *J* = 1.5, 7	—
H-15β	1.75 (m)	1.70 (m)	1.80 (m)	1.84 (m)	4.12 (dt) *J* = 3.4, 9.6	—	1.78 (m)	—	—	4.08 (dt) *J* = 3.7, 9.4
H-16α	1.33 (m)	1.36 (m)	1.41 (m)	1.38 (m)	1.32 (m)	1.93 (dd) *J* = 7, 14.9	1.38 (m)	1.89 (dd) *J* = 7.4, 14	1.98 (dd) *J* = 7.2, 15.6	1.33 (dd) *J* = 3.7, 14.5
H-16β	2.76 (m)	2.66 (m)	2.68 (m)	2.70 (m)	3.24 (dd) *J* = 9.6, 14	2.78 (d) *J* = 14.9	2.69 (m)	2.85 (d) *J* = 16	2.80 (m)	3.19 (dd) *J* = 9.6, 14.5
18-CH_3	0.81 (s)	1.00 (s)	0.80 (s)	0.84 (s)	0.82 (s)	1.06 (s)	0.87 (s)	1.39 (s)	1.13 (s)	1.05 (s)
H21	4.41 (q) *J* = 6.4	4.42 (q) *J* = 6.8	4.40 (q) *J* = 6.4	4.43 (q) *J* = 6.4	4.38 (q) *J* = 6.4	4.40 (q) *J* = 6.4	4.43 (q) *J* = 6.4	4.39 (q) *J* = 6.3	4.41 (q) *J* = 6.4	4.46 (q) *J* = 6.4
22-CH_3	1.30 (d) *J* = 6.4	1.31 (d) *J* = 6.8	1.31 (d) *J* = 6.4	1.33 (d) *J* = 6.4	1.29 (d) *J* = 6.4	1.35 (d) *J* = 6.4	1.33 (d) *J* = 6.4	1.32 (d) *J* = 6.3	1.34 (d) *J* = 6.4	1.25 (d) *J* = 6.4
23-CH_3	1.15 (s)	1.12 (s)	1.21 (s)	1.17 (s)	1.28 (s)	1.12 (s)	1.17 (s)	1.08 (s)	1.16 (s)	1.27 (s)

Table 3. Complete assignments of ^{13}C NMR spectra of **RU27987** and metabolites **2**–**10** (δ ppm; 125.77 MHz in $CDCl_3$). Parenthesised numbers correspond to observed multiplicities (as number of hydrogens attached) as determined by DEPT experiments

Carbon	RU27987	2	3	4	5	6	7	8	9	10
1	25.6 (2)	25.0 (2)	26.2 (2)	64.5 (1)	25.8 (2)	25.7 (2)	25.9 (2)	25.6 (2)	27.0 (2)	26.2 (2)
2	37.0 (2)	36.7 (2)	37.3 (2)	45.3 (2)	36.2 (2)	36.9 (2)	37.2 (2)	35.9 (2)	35.0 (2)	37.5 (2)
3	199.6 (0)	199.7 (0)	197.7 (0)	196.7 (0)	199.8 (0)	199.6 (0)	200.1 (0)	199.2 (0)	191.4 (0)	204.1 (0)
4	122.3 (1)	124.4 (1)	123.7 (1)	121.8 (1)	122.1 (1)	121.7 (1)	121.9 (1)	129.5 (1)	130.5 (1)	123.7 (1)
5	156.9 (0)	157.2 (0)	157.3 (0)	152.7 (0)	157.1 (0)	156.9 (0)	155.1 (0)	155.8 (0)	157.2 (0)	160.4 (0)
6	30.8 (2)	30.6 (2)	30.7 (2)	29.6 (2)	30.8 (2)	30.8 (2)	68.5 (1)	26.5 (2)	25.7 (2)	31.1 (2)
7	27.7 (2)	27.3 (2)	27.1 (2)	26.1 (2)	27.6 (2)	27.1 (2)	34.2 (2)	36.8 (2)	19.5 (2)	28.2 (2)
8	39.5 (1)	35.8 (1)	38.7 (1)	38.9 (1)	39.0 (1)	35.3 (1)	34.5 (1)	73.1 (0)	31.8 (1)	36.7 (1)
9	145.4 (0)	145.0 (0)	145.4 (0)	151.2 (0)	144.9 (0)	145.3 (0)	145.0 (0)	142.3 (0)	68.4 (0)	147.0 (0)
10	125.7 (0)	130.1 (0)	129.1 (0)	128.9 (0)	126.0 (0)	125.9 (0)	122.7 (0)	127.5 (0)	59.7 (0)	130.3 (0)
11	25.8 (2)	66.0 (1)	70.5 (1)	25.9 (2)	25.5 (2)	25.4 (2)	25.7 (2)	23.1 (2)	24.9 (2)	66.1 (1)
12	32.9 (2)	39.8 (2)	43.9 (2)	33.4 (2)	33.0 (2)	33.8 (2)	33.0 (2)	34.4 (2)	32.6 (2)	40.8 (2)
13	45.2 (0)	44.4 (0)	44.8 (0)	44.8 (0)	45.6 (0)	44.7 (0)	45.4 (0)	45.2 (0)	45.0 (0)	45.9 (1)
14	51.1 (1)	50.0 (1)	49.8 (1)	49.6 (1)	57.3 (1)	55.2 (1)	50.4 (1)	55.6 (1)	50.5 (1)	57.0 (1)
15	24.1 (2)	23.7 (2)	23.8 (2)	24.4 (2)	73.0 (1)	69.5 (1)	24.0 (2)	71.1 (1)	69.0 (1)	73.1 (1)
16	30.9 (2)	30.4 (2)	30.8 (2)	30.7 (2)	42.6 (2)	44.0 (2)	30.7 (2)	44.1 (2)	44.3 (2)	43.5 (2)
17	60.1 (0)	60.5 (0)	59.5 (0)	59.7 (0)	58.2 (0)	60.0 (0)	59.9 (0)	60.3 (0)	59.7 (0)	59.7 (0)
18	15.8 (3)	17.9 (3)	17.4 (3)	15.8 (3)	17.1 (3)	18.1 (3)	16.0 (3)	19.6 (3)	18.3 (3)	19.5 (3)
20	217.2 (0)	217.7 (0)	217.3 (0)	217.0 (0)	216.4 (0)	216.2 (0)	217.0 (0)	216.0 (0)	215.9 (0)	218.9 (0)
21	69.8 (1)	69.7 (1)	69.6 (1)	69.6 (1)	69.6 (1)	69.7 (1)	69.3 (1)	69.8 (1)	69.5 (1)	69.9 (1)
22	22.2 (3)	22.0 (3)	22.0 (3)	22.1 (3)	22.1 (3)	22.2 (3)	22.2 (3)	22.1 (3)	21.9 (3)	22.0 (3)
23	21.6 (3)	21.8 (3)	21.7 (3)	21.5 (3)	21.3 (3)	22.0 (3)	21.8 (3)	21.0 (3)	21.1 (3)	20.7 (3)

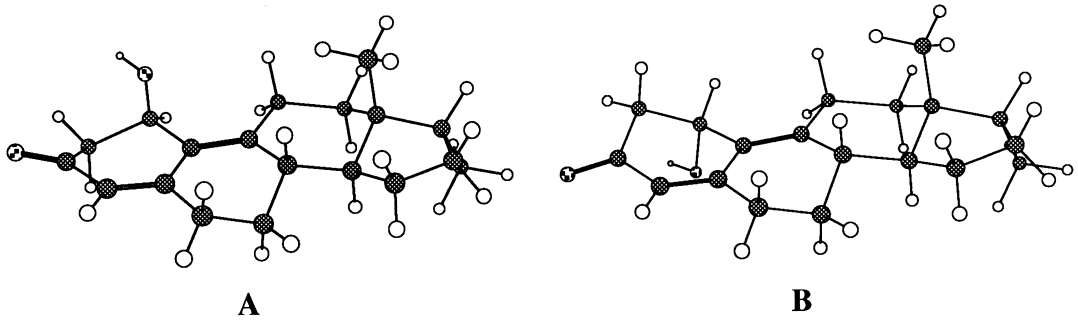

Figure 3. Minimized conformations of 1β-hydroxy (A) and 1α-hydroxytrimegestone (B).

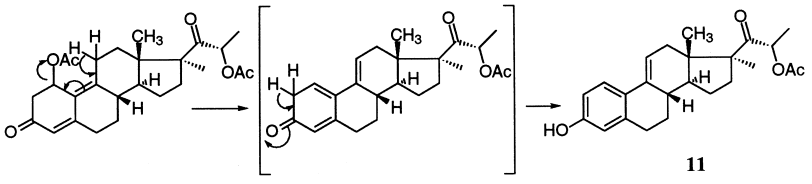

Figure 4. Mechanism for aromatization of ring A and formation of the phenolic derivative **11** from the acetylation product of 1-hydroxytrimegestone **4**.

LCP 57.1569, *A. alliaceus* NRRL 315, *C. minor* MMP 1197 or *S. racemosum* LCP 72.2150 (see Table 1). It was obtained in 30% isolated yield from a 24-h incubation of **RU27987** with *A. corymbifera* (50% yield by HPLC). The ^1H and ^{13}C NMR data of **6** were very similar to those of the 15α-hydroxy derivative **5**, except for a significant downfield shift of H-8 ($\Delta\delta = +0.2$ ppm) and upfield shifts of H-16α ($\Delta\delta = -1.27$ ppm) and H-14 ($\Delta\delta = -0.17$ ppm) signals, associated with a smaller coupling constant between the latter and the -C*H*(OH)- signal (about 6 versus 9 Hz), and a downfield shift of the 18-CH_3 signal ($\Delta\delta = +0.24$ ppm). Metabolite **6** is thus undoubtedly the 15β-hydroxy stereoisomer of **5**, and this structure was entirely confirmed by an exhaustive examination of homo- and heteronuclear correlation spectra. No interconversion between **5** and **6** could be observed in the incubation mixtures, as the possible result of oxido-reduction reactions.[26]

Metabolite **7** was formed in significant amounts only by a few strains such as *F. roseum* ATCC 14717 and *M. isabellina* MMP 52.108 (about 15% by HPLC). It was obtained, together with metabolite **5**, from an incubation with *F. roseum* (11% isolated yield). ^1H and ^{13}C NMR data of this monohydroxylated metabolite of **RU27987** showed again the replacement of a -CH_2- group by a -CH(OH)- group. Accounting for the assigned locations of the hydroxyl group in previously

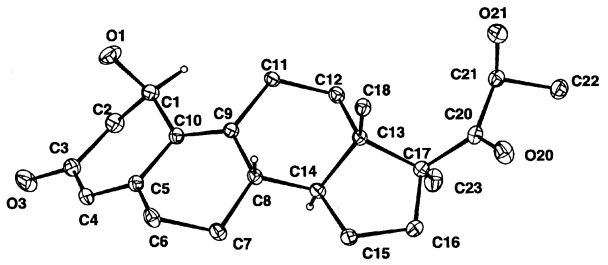

Figure 5. ORTEP drawing of metabolite **4**.

identified metabolites, and the unchanged chemical shifts of 18-CH_3 and C-17 methyl signals, only positions -1, -2, -6, or -7 remain possible. A location on 1- or 2-positions was ruled out by the absence of correlation of the -C*H*(OH)- signal with C-3, as shown by HMBC experiments. Moreover, the coupling of this signal with only two protons, revealed by ^{1}H-^{1}H COSY correlation spectra, is consistent with the structure of a 6-hydroxy derivative of **RU27987**. The stereochemistry of this hydroxyl group was not easy to determine owing to the flexible conformation of the C-6-C-7 bond; however the coupling pattern of the 7-hydrogens, using H-6 and H-8 as reporter signals, was only compatible with a 6β-hydroxy derivative, in the minimal energy conformation[24] shown in Figure 6, where *eq*-H-7β exhibits two small ^3J coupling constants with H-6 and H-8 (about 5 Hz), while *ax*-H-7α exhibits a large ^3J constant (9 Hz) with H-8 and a small one (2.8 Hz) with H-6, as determined by selective irradiation experiments. NOESY studies confirmed the assigned 6β-OH configuration and conformation of Figure 6 by showing a strong nuclear Overhauser effect between H-6α and H-4 (2.39 A), a very similar NOE between H-6α and both H-7 hydrogens (2.45 and 2.48 A), and a selective NOE between H-8 and H-7β (2.45 A) without any significant effect on H-7α (3.09 A).

Metabolite **8** was never formed as a major metabolite. However, it was obtained in a moderate amount (15–20% by HPLC, 8% isolated yield) from a prolonged incubation with *A. corymbifera* LCP 63.1800, together with **6**. The kinetics of formation of metabolite **8** was correlated with the decrease of **6** (see Fig. 2), suggesting an overoxidation of the 15β-hydroxylated metabolite. ^{1}H and ^{13}C NMR data of this dihydroxylated metabolite of **RU27987** (MH^+ at *m/z* 375) showed the replacement of a -CH- group by a quaternary C–OH and the replacement of a -CH_2- group by a -CH(OH)-group. 18-CH_3 localized by HMQC experiments was unusually shifted to higher frequencies ($\Delta\delta = +0.58$ ppm) indicating strong 1,3-diaxial interaction(s) with β-OH substituent(s) at positions 11β, 8β, 15β and/or 16β. An interaction with an -OH group in position 12 could not be also eliminated. As the resonance of the methyl group protons on C-17 was not affected, position 16 was excluded. Coupling of the new -CHOH- proton with 3 hydrogens eliminated positions 11 and 12, emphasizing a localization at C-15β which was confirmed by a clear coupling with the C-14 hydrogen, appearing as a simple doublet ($J = 5.6$ Hz) as the result of C-8 hydroxylation, the only remaining possibility of a tertiary C-hydroxylation. All other assignments deduced from ^{1}H-^{1}H COSY spectra and HMBC and HMQC experiments correlated perfectly with the proposed structure of a 8β,15β-dihydroxy derivative of **RU27987**.

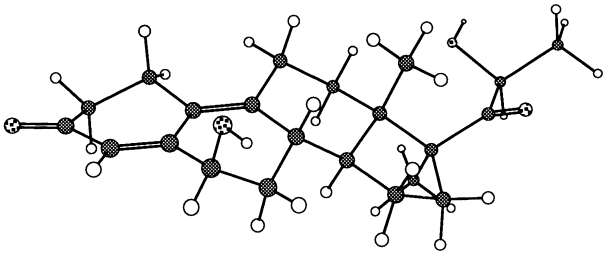

Figure 6. Minimal energy conformation of 6β-hydroxytrimegestone (metabolite **7**).

Metabolite **9** was isolated in small amount (5% isolated yield) from a 48-h incubation of **RU27987** with *C. echinulata* ATCC 9244, together with **2**, **4** and **6**. A delayed formation of this metabolite, a high polarity, and mass spectral data which indicated the introduction of two new oxygen atoms in the molecule (MH^+ at $m/z = 375$), suggested again the structure of a secondary oxidation metabolite deriving from one of the main initial products. A UV-absorbance maximum shifted to 250 nm indicated the loss of the $\Delta^{9,10}$ double bond, while the presence of the original H-4 singlet at 6.06 ppm showed the preservation of the Δ^4-conjugated double bond. Compared to **RU27987**, the occurrence of an additional -CH(OH)- in place of a -CH_2- group, and the same number of quaternary carbon atoms in ^{13}C NMR, suggested a 9,10-epoxy hydroxylated derivative. A comparison of NMR data, and particularly ^{1}H NMR assignments of H-15, H-16, 18-CH_3 and CH_3 on C-17 with those of hydroxylated accompanying metabolites pointed up the similarity with metabolite **6**. All other assignments were easily deduced from ^{1}H-^{1}H COSY spectra and HMBC or HMQC experiments. The significant deshielding of H-14 at 1.91 ppm ($\Delta\delta = +0.37$ ppm) and that of one of the hydrogens in position -7 ($\Delta\delta = +0.24$ ppm) suggested an α-stereochemistry for the oxirane ring. Metabolite **9** was thus tentatively assigned the structure of a 9α,10α-epoxy-15β-hydroxy derivative of **RU27987**.

Metabolite **10** was obtained in 25% yield from an incubation of **RU27987** with *S. rimosus* NRRL 2234. Metabolite **2** was primarily formed during the first 48 h of incubation, then it was slowly and nearly completely converted to metabolite **10** after 96 h. Metabolite **10** was a dihydroxylated derivative of **RU27987** (MH^+ at *m/z* 375), resulting from the hydroxylation of two -CH_2- groups, as shown by ^{1}H and ^{13}C NMR. In the ^{1}H NMR spectra, 18-CH_3 was shifted to higher frequencies ($\Delta\delta = +0.24$ ppm), indicating a strong 1,3-diaxial interaction with a β-OH substituent at position 11β, 15β or 16β. Kinetic data of the conversion of initially formed 11β-hydroxy trimegestone (**2**) (Fig. 2) and comparison of ^{1}H NMR data of **2** and **10** confirmed the presence of a hydroxyl group at position 11β. The signal of the methyl group borne by C-17 was significantly

downfield shifted ($\Delta\delta = +0.12$ ppm), suggesting an additional hydroxylation in position 15α or 16. A careful examination of the ^{1}H-^{1}H COSY correlation spectra revealed a coupling of this -CH(OH)- group (at 4.08 ppm) with three protons, establishing a 15α position for this hydroxyl group. A comparison of ^{1}H and ^{13}C NMR data with those of previously isolated metabolites (and particularly 15α-hydroxytrimegestone **5**) strongly suggested again an 11β,15α-dihydroxylated derivative of **RU27987**. All other assignments deduced from HMBC and HMQC experiments correlated perfectly with the proposed structure.

Discussion

It was surprising to note that more than half of fungi or *Streptomyces* strains tested (29 out of 51) were able to actively transform **RU27987**. The major metabolite observed in most strains (Table 1) was the 11β-hydroxy derivative, corresponding to the only previously reported product for the microbial metabolism of ketodiene substrates by *C. lunata*.[20] Apparently, such an 11-hydroxylation is particularly facilitated, while hydroxylation at 6- and 1-positions, also in activated allylic locations, are much less favored. The 11α-hydroxylated metabolite is occasionally formed by some strains, but is apparently less stable, as it can isomerize spontaneously to the 11β-isomer, without the intermediate formation of the 11-keto derivative, which was never detected. Interestingly, none of the 11-hydroxylated metabolites have been found in the human metabolism of Trimegestone. Concerning other hydroxylated derivatives, only a few strains, such as *M. isabellina* or *R. arrhizus*, produce complex product mixtures. Usually, a limited number of metabolites, differing with the strain used, predominates. The variety of other hydroxylated compounds isolated, sometimes in high conversion yields (45–70% isolated products), may match with the usual steroid hydroxylation sites already described for the corresponding strains: for example *Fusarium roseum* has been previously described to give mainly 15α- and 6β-hydroxylations of 19-nor-steroids,[27] in agreement with that found in the present work. However, 7α- and 14α-hydroxylated derivatives, frequently found in the hydroxylation of the current steroid substrates by various 11β-hydroxylating strains (*Mucor griseocyanus* for example,[21,28–30]), are not produced from **RU27987**. This result is possibly related to a more stringent orientation of this substrate in the active site of the monooxygenase(s) involved,[31] prohibiting the "capsizing" (or "back-to-front" binding) of the steroid ring, put forward for example in the non-regiospecific hydroxylations of progesterone and related steroids by *C. lunata*[20] or *Cochliobolus lunatus*.[32] This may be also related to the peculiar shape of the ketodiene-constrained AB-ring system, which has been previously referred to for explaining the high affinity of this steroid type for the progesterone receptor.[22,23]

There is no evidence that more than one steroid monooxygenase exists in the strains used, at least in the initial monohydroxylation reactions. Contradictory results have been reported: the mutagenic treatment of uninucleated protoplasts of *Curvularia lunata* IM 2901 afforded mutants with decreased by-product formation and significant increase of 11β-hydroxylation of progesterone; however, no evidence about the operation of highly directional independent enzymes or a single enzyme with diminished specificity could be inferred from these results.[33] In our laboratory, an exhaustive screening of mutants obtained from *C. echinulata* by similar techniques could not show any significant alteration in the ratio of products formed from **RU27987** by this strain (V. de Oliveira and R. Azerad, unpublished results).

On the other hand, it is not clear whether overoxidation products such as the epoxide **9** or dihydroxylated metabolites **8** and **10**, the formation of which is generally delayed, are generated by the same enzyme, acting on the primary hydroxylation product when its concentration increases in the incubation medium. A possible induction of new monooxygenase(s) in the late phase of the transformation can be responsible for the formation of such products. This question remains open and should be elucidated only on isolation and purification of the microbial monooxygenase(s) involved.

Two of the microbial metabolites, the 1β-hydroxy and the 6β-hydroxy derivatives (metabolites **4** and **7**), were found to be produced in small amounts by human healthy volunteers: in this case, the possession of authentic specimens of these metabolites, prepared by microbial methods, although in low yields, represented an invaluable facility for the identification of the human metabolites by chromatographic methods. All previous attempts to prepare metabolite **4** by chemical methods have invariably failed.

In addition, most of the microbial metabolites described were obtained in sufficient amounts to be assayed in activity test systems. As the steroid flexibility, which is probably modified in hydroxylated derivatives, seems to determine the specificity of the biological response,[22] the pharmacological profile of these metabolites has been studied both in vitro and in vivo. In vitro assays were performed with human recombinant hormonal receptors[34] and main results for two metabolites expressing sufficient activities on these receptors are given in Table 4. Metabolite **4** (1β-hydroxytrimegestone) exhibited good relative binding affinity (RBA) for the human progestogen receptor, although this was nine times weaker than that of **RU27987**. Metabolite **7** (6β-hydroxytrimegestone) only showed a moderate RBA for this receptor. Contrarily to **RU27987** which showed moderate RBAs for the human glucocorticoid and mineralocorticoid receptors, and a weak RBA for the human androgen receptor, these two metabolites showed no or negligible RBAs for these receptors. They thus present a higher selectivity for the human progestogen receptor.

The in vivo progestomimetic activity of these two metabolites has been investigated by the endometrial transformation in the rabbit. In this test, known as the Clauberg–McPhail test,[34,35] metabolite **4** was assayed by subcutaneous route and orally, whereas metabolite **7** and progesterone, as a reference, were administered by

Table 4. Relative binding affinities (RBAs) for Trimegestone and metabolites **4** and **7**. The RBAs of respective reference ligands (dexamethasone, progesterone, testosterone, aldosterone and estradiol) were arbitrarily taken to be equal to 100

Ligands	Human glucocorticoid receptor (24 h at 0°C)	Human progestogen receptor (24 h at 0°C)	Human androgen receptor (24 h at 0°C)	Human mineralocorticoid receptor (24 h at 0°C)	Human estrogen receptor (24 h at 0°C)
RU27987	14	588	2.5	28	0
Metabolite **4** (1β-hydroxy-Trimegestone)	0.06	64	0.04	0.1	0
Metabolite **7** (6β-hydroxy-Trimegestone)	0.05	12	0	0.3	0

subcutaneous route (progesterone is inactive when administered by the oral route). The Clauberg–McPhail test (Table 5) showed a good progestomimetic activity for metabolite **7** at 1 mg/kg. Metabolite **4** at the starting dose of 0.01 mg/kg by subcutaneous route showed the maximal activity, as progesterone at 0.1 mg/kg; at a dose of 1 mg/kg per os, activity expressed was better than by subcutaneous route. Furthermore, histological observation of the uterus did not reveal any morphological anomaly.

In conclusion, the microbial generation of metabolites from **RU27987** has constituted a powerful and versatile tool for the identification of its human and animal metabolites. In addition, this method allowed the production of new, active and potentially useful hydroxylated derivatives, which would have been accessed with difficulty by chemical synthesis.

Experimental

General. ^{1}H and ^{13}C NMR (1-D and 2-D) spectra were performed at 500.13 and 125.77 MHz, respectively, on a Bruker AMX500-2 instrument, using standard pulse sequences. Chemical ionization (NH_3) and high-resolution mass spectrometric analyses were performed at the Mass Spectrometry Service of the Ecole Normale Supérieure (Paris). UV spectra were recorded with a Uvikon 810 spectrophotometer (Kontron). Melting points were determined in capillary tubes with a Büchi instrument and are uncorrected. Optical rotations were measured using a Perkin–Elmer 241C spectropolarimeter, in a 1 dm cell.

Table 5. In vivo activity in the Clauberg–McPhail test (endometrial proliferation in the rabbit). After treatment, animals were sacrificed and a median portion of each uterine horn was prepared for histological examination under light-optical microscopy. A semi-quantitative evaluation of endometrial proliferation (uterine lace) was carried out by the MacPhail scale from 0 to 4 with 0.5 intermediates and the two figures were averaged. The degree of horn hypertophy was evaluated by the same scale. S/c: Subcutaneous route; per os: oral route. Progesterone and Trimegestone were used as reference products

Test compounds	Concentration (mg/kg)	Administration route	Average McPhail index
Progesterone	0.1	S/c	1.8
	1	S/c	3.8
Trimegestone **1**	0.003	S/c	3.8
Metabolite **7** (6β-hydroxytrimegestone)	0.01	S/c	1.3
	0.1	S/c	2
	1	S/c	3.8
Metabolite **4** (1β-hydroxytrimegestone)	0.01	S/c	3.8
	0.1	S/c	3.8
	1	S/c	3.8
	1	per os	4

Microorganisms. All fungal cultures were maintained on agar slants containing (per litre), yeast extract (Difco) 5 g, malt extract (Difco) 5 g, glucose 20 g and Bacto-agar (Difco) 20 g, stored at 4°C and subcultured at 26°C before use. Fungi were purchased from the American Type Culture Collection (ATCC strains), Rockville, MD, USA, the Northern Regional Research Laboratories (NRRL strains), Peoria, IL, USA, the Centralbureau voor Schimmelcultures (CBS strains), Baarn, the Netherlands, or the Laboratoire de Cryptogamie of the Museum d'Histoire Naturelle (LCP strains), Paris, France. Some strains (MMP or BO) are from local origin. *Streptomyces* strains (purchased from the same collections) were maintained on agar slants containing (per litre), yeast-malt extract agar (Difco) 38 g, stored at 4°C and subcultured at 26°C before use.

Chromatographic procedures. Analytical reverse-phase HPLC was carried out using the following conditions: column, Nucleosil 5C18 (250×4.6 mm); solvent, MeOH in water (linear gradient from 50 to 65% during 25 min, then 65% during 25 min), 0.8 mL/min, using Gilson 305 pumps, a Gilson 231 sample injector equipped with a 20 μL loop, a Shimadzu SPD-6A LC-UV detector set at 310 or 244 nm and a Shimadzu C-R6A integrator recorder. Calibration was performed using external standard solutions of **RU27987** or 11β-hydroxytrimegestone (metabolite **2**). When necessary, some separations were performed on a preparative scale (20–50 mg), using a Nucleosil C18 (100 A, 10 μm, 250×22 mm) column with a MeOH-water solvent (20 mL/min). TLC was carried out with the ascending method using silica gel $60F_{254}$ precoated glass plates (Merck, Germany). Spots were detected under UV light and by spraying with a 5% solution of phosphomolybdic acid in 3 M H_2SO_4.

Substrates and reference compounds. Trimegestone® (**RU27987**) was kindly donated by HMR France. Other HMR synthetic compounds such as 11β-hydroxytrimegestone (**2**) and several oxidized derivatives were

used for chromatographic comparisons. All other chemicals were obtained from commercial sources and used without further purification.

Microbial transformations

Culture and screening procedures. Fungi were grown at 27°C in a liquid medium containing (per litre), corn steep liquor (Roquette, France) 10 g, glucose 30 g, KH_2PO_4 1 g, K_2HPO_4 2 g, $NaNO_3$ 2 g, KCl 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.02 g. *Streptomyces* strains were grown at 27°C in a liquid medium containing (per litre) yeast extract (Difco) 5 g, soy peptone (Organotechnie, France) 5 g, glucose 20 g, NaCl 5 g, K_2HPO_4 5 g and adjusted to pH 7 with HCl.

For screening experiments, 250 mL conical flasks containing 100 mL (fungi) or 50 mL (*Streptomyces*) of sterile liquid medium were inoculated with a few drops of a spore suspension obtained from freshly grown agar slants, then orbitally shaken (200 rpm) at 27°C for 60–65 h. The substrates were then added as ethanol solutions (1 mL) to yield a final concentration of 0.5 g/L. Samples (1–2 mL) were aseptically withdrawn every day, centrifuged and the supernatants were microfiltered (0.45 μm). Aliquots of the filtrates were analyzed by reverse-phase HPLC, and the remaining solution was saturated with sodium chloride and extracted with ethyl acetate for TLC analysis. Most transformations were continued up to 7 days or until no further increase of metabolite(s) was observed. Control experiments performed by incubating fungi or *Streptomyces* strains in the absence of substrate were currently run to exclude mycelium products possibly detected by HPLC.

General preparative biotransformation procedure. Extrapolation of screening methods to a larger number of flasks or larger volume incubation was performed for the preparation of every metabolite, using the best strain previously selected. The bioconversions were monitored by HPLC determination of the desired metabolites in the incubation supernatant, as described above, and stopped by filtration of the biomass, saturation of the filtrate with sodium chloride and repeated extraction with ethyl acetate. After drying with $MgSO_4$, the extract was evaporated in vacuo and the oily crude product was flash-chromatographed on a silica gel column to give pure or enriched fractions. Final purification was achieved by crystallisation, thin layer chromatography or preparative reverse phase HPLC.

Metabolites of RU27987

Preparation of metabolite 2 (11β-hydroxytrimegestone). *Mucor hiemalis* BO was grown during 66 h in three 2 L-Erlenmeyer flasks containing 3×800 mL of liquid medium. **RU27987** (300 mg) dissolved in ethanol (8 mL) was added to each flask and incubated at 27°C with orbital shaking (200 rpm) during 3 days. The incubation mixture was filtered and the filtrate was repeatedly extracted with ethyl acetate to give 880 mg of a crude yellow oil. After chromatography on a silica gel column, using EtOAc:CH_2Cl_2 (8:2) as solvent, 589 mg of **2** (63%) were obtained. Mp 162°C. HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2227. $[\alpha]_D$ +44.8° (*c* 0.97, $CHCl_3$). NMR, see Tables 2 and 3.

Preparation of metabolites 3 (11α-hydroxytrimegestone) and 6 (15β-hydroxytrimegestone). *Absidia cylindrospora* LCP 57.1569 was grown for 66 h in a 2 L-Erlenmeyer flask containing 1 L of liquid medium. **RU27987** (500 mg) dissolved in ethanol (6 mL) was added and incubation was continued at 27°C with orbital shaking (200 rpm) during 24 h. Filtration of the incubation mixture, saturation of the filtrate with sodium chloride and repeated extraction with ethyl acetate afforded a crude yellow oil (560 mg). After chromatography on a silica gel column using CH_2Cl_2:*i*PrOH (95:5) as solvent, 99 mg of **2**, 29 mg of **3** and 162 mg of **6** were obtained.

Pure metabolite **3** (10 mg) was obtained by purification on a silica gel thin layer plate using EtOAc:MeOH (50:50) as solvent. Mp 178–183°C dec. HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2224. $[\alpha]_D$ −18.7° (*c* 0.99, $CHCl_3$). NMR, see Tables 2 and 3.

Pure metabolite **6** (108 mg) was obtained by crystallisation in EtOAc:cyclohexane. Mp 160−162°C. HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2239. $[\alpha]_D$ −109.4° (*c* 0.78, $CHCl_3$). NMR, see Tables 2 and 3.

In a parallel experiment, a grown culture of *Circinella minor* was incubated for 3 days with **RU27987** (0.5 g/L), producing metabolite **6** (26%), metabolite **3** (23%) and metabolite **2** (15%) as estimated by HPLC of the incubation mixture. After heating the filtrate (pH 6) at 60°C for 1 h, HPLC showed a nearly complete conversion of metabolite **3** (2%) into metabolite **2** (36%).

Preparation of metabolites 4 (1β-hydroxytrimegestone) and 9 (9α,10α-epoxy-15β-hydroxytrimegestone). *Cunninghamella echinulata* var. *echinulata* ATCC 9244 (= *C. bainieri*) was grown for 65 h in two 2 L-Erlenmeyer flasks containing each 0.8 L of liquid medium. **RU27987** (400 mg) dissolved in ethanol (5 mL) was added to each flask and incubated at 27°C with orbital shaking (200 rpm) during 3 days. The incubation mixture was filtered and the filtrate, saturated with sodium chloride, was repeatedly extracted with ethyl acetate, affording 965 mg of a yellowish oil which was chromatographed on a silica gel column using CH_2Cl_2:*i*PrOH (95:5) as solvent. Beside fractions containing mixtures of metabolites, one fraction containing pure metabolite **9** (45 mg) was obtained. Mp 87–90°C dec. HRMS (CI, CH_4), calcd for $C_{22}H_{31}O_5$ (MH^+), 375.2171; found 375.2169. $[\alpha]_D$ −50.0° (*c* 1.5, MeOH). NMR, see Tables 2 and 3.

Pure metabolites **2** (82 mg) and **4** (85 mg) were obtained by preparative HPLC (solvent MeOH:water, 1:1) from another fraction (209 mg). Metabolite **4**: Mp 80–85°C (after crystallisation from CH_2Cl_2:EtOAc). HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2231. $[\alpha]_D$ +80.2° (*c* 1.06, $CHCl_3$). NMR, see Tables 2 and 3.

A sample of metabolite **4** (60 mg) was treated overnight with excess acetic anhydride in pyridine at room temperature. After extraction and washing, the crude product was purified on a thin layer plate and crystallised in acetone:EtOAc to give **11** (40 mg). Mp 245°C dec. ^{1}H NMR (DMSO, 250 MHz), δ 9.28 (1H, s, OH), 7.47 (1H, d, $J=8.7$, H-1), 6.59 (1H, dd, $J=2.4$, 8.4, H-2), 6.50 (1H, d, $J=2.4$, H-4), 6.10 (1H, dd, $J=2.4$, 3.9, H-11), 5.41 (1H, q, $J=6.4$, H-21), 2.10 (3H, s, $COCH_3$), 1.36 (3H, d, $J=6.4$, CH_3-22), 1.21 (3H, s, CH_3-23), 0.66 (3H, s, CH_3-18).

Preparation of metabolites 5 (15α-hydroxytrimegestone) and 7 (6β-hydroxytrimegestone). *F. roseum* ATCC 14717 was grown for 65 h in two 250 mL-Erlenmeyer flasks containing each 100 mL of liquid medium. **RU27987** (50 mg) dissolved in ethanol (1 mL) was added to each flask and incubated at 27°C with orbital shaking (200 rpm) during 2 days. The incubation mixture was filtered and the filtrate, saturated with sodium chloride, was repeatedly extracted with ethyl acetate, affording 116 mg of a yellow oil which was deposited on two thin layer silica gel plates and chromatographed with CH_2Cl_2:*i*PrOH (9:1) as solvent. Elution of the major bands by EtOAc:MeOH (1:1) gave 47 mg of metabolite **5**, mp 155–160°C. HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2225. $[\alpha]_D$ +106.6° (*c* 0.8, $CHCl_3$). NMR, see Tables 1 and 2 and 11.5 mg metabolite **7**, mp 73–75°C. HRMS (CI, NH_3), calcd for $C_{22}H_{31}O_4$ (MH^+), 359.2224; found 359.2231. $[\alpha]_D$ −122.1° (*c* 0.86, $CHCl_3$). NMR, see Tables 2 and 3.

Preparation of metabolites 6 (15β-hydroxytrimegestone) and 8 (8β,15β-dihydroxytrimegestone). *Absidia corymbifera* LCP 63.1800 was grown for 65 h in five 250 mL-Erlenmeyer flasks containing each 100 mL of liquid medium. **RU27987** (50 mg) dissolved in ethanol (1 mL) was added to each flask and incubated at 27°C with orbital shaking (200 rpm) during 7 days. The incubation mixture was filtered and the filtrate, saturated with sodium chloride, was repeatedly extracted with ethyl acetate, affording 269 mg of a yellowish oil which was dissolved in methanol and separated by preparative HPLC (solvent MeOH:water, 45:55) to give the known metabolite **6** (85 mg) and 21 mg of metabolite **8**. Mp 180–190°C. HRMS (CI, NH_3): calcd for $C_{22}H_{31}O_5$ (MH^+), 375.2171; found 375.2173. $[\alpha]_D$ −127.0° (*c* 0.37, MeOH). NMR, see Tables 2 and 3.

Preparation of metabolite 10 (11β,15α-dihydroxytrimegestone). *S. rimosus* NRRL 2234 was grown for 66 h in a 250 mL-Erlenmeyer flask containing 100 mL of liquid medium. **RU27987** (50 mg) dissolved in ethanol (1 mL) was added and incubation was continued at 27°C with orbital shaking (200 rpm) during 4 days. Filtration of the incubation mixture, saturation of the filtrate with sodium chloride and repeated extraction with ethyl acetate afforded 25 mg of a crude yellow oil from which metabolite **10** (12 mg) was isolated and purified by preparative TLC (solvent EtOAc:CH_2Cl_2, 9:1). Mp 115–120°C. HRMS: calcd for $C_{22}H_{31}O_5$ (MH^+), 375.2171; found 375.2181. NMR, see Tables 2 and 3.

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