Nonactin Biosynthesis: On the Role of (6*R*,8*R*)- and (6*S*,8*S*)-2-Methyl-6,8-dihydroxynon-2*E*-enoic Acids in the Formation of Nonactic Acid

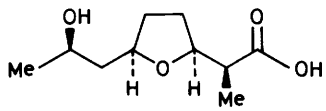
Zoe M. Spavold and John A. Robinson

Chemistry Department, The University, Southampton SO9 5NH, U.K.

Enantiospecific syntheses of (6*R*,8*R*)- and (6*S*,8*S*)-2-methyl-6,8-dihydroxynon-2*E*-enoic acids and their corresponding caprylcysteamine thiol esters are described; these thiol esters are incorporated efficiently and enantiospecifically into nonactin, thereby establishing the direct precursor role of these units in nonactin biosynthesis.

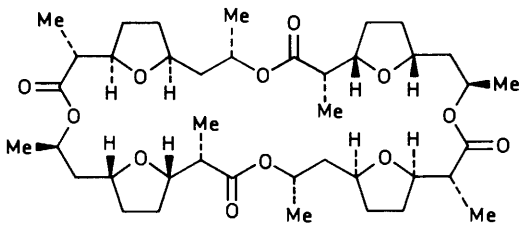
Important questions regarding the role, if any, of presumptive intermediates in the biosynthesis of the macrolide antibiotics erythromycin and tylosin have been addressed for the first time recently, by administering labelled forms of carbon backbone fragments, activated as thiol esters, to producing cultures of the relevant micro-organism.[1] We have also used this technique to study the biosynthesis of the macrotetrolide family of ionophore antibiotics, products of current interest to the agrochemical industry for their antiparasitic and miticidal activity.[2] Our objective has been to identify product(s) of the presumptive synthase responsible for constructing the carbon backbones of (+)- and (−)-nonactic acids (**1**) from primary precursors, and to identify specific reactions which introduce chirality into this backbone, for use in assays of the corresponding biosynthetic enzymes in cell free systems. The incorporations of ^{13}C and ^{18}O labelled acetate and propionate[3] into nonactin (**2**) implicated the diketone (**3**) and diols (**4**) and (**5**) as potential precursors of the activated nonactic acids (**6**) and (**7**) (see Scheme 1). We have, therefore, sought to test directly the precursor roles of diols (**4**) and (**5**), and report here the synthesis of these materials activated as caprylcysteamine thiol esters, and their incorporation into nonactin in whole cell cultures of *Streptomyces griseus*.

The synthetic chemistry employed to prepare these diols is outlined in Scheme 2. Thus the preparation of the epoxy-alcohols (**8**) in optically pure form (>95% enantiometric excess) was achieved by the procedure described by Sutherland *et al.*[4] From this, both the free acids (**9**) and (**10**), and the thiol esters (**11**) and (**12**) can easily be prepared in labelled form,† suitable for incorporation experiments. The stereo-

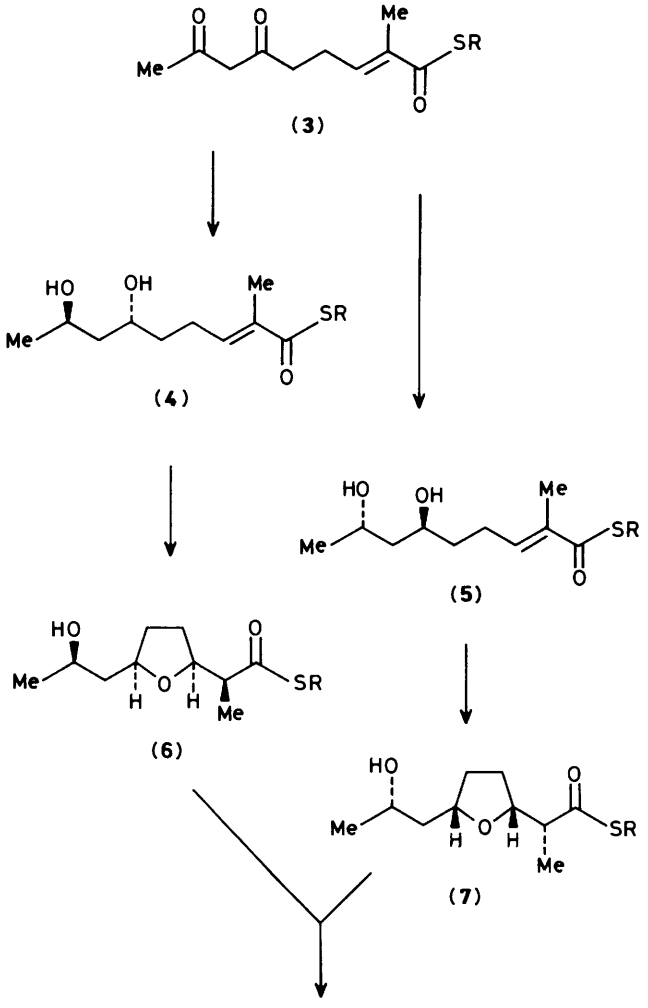


(+)-(2*S*,3*S*,6*R*,8*R*)-nonactic acid (**1**)

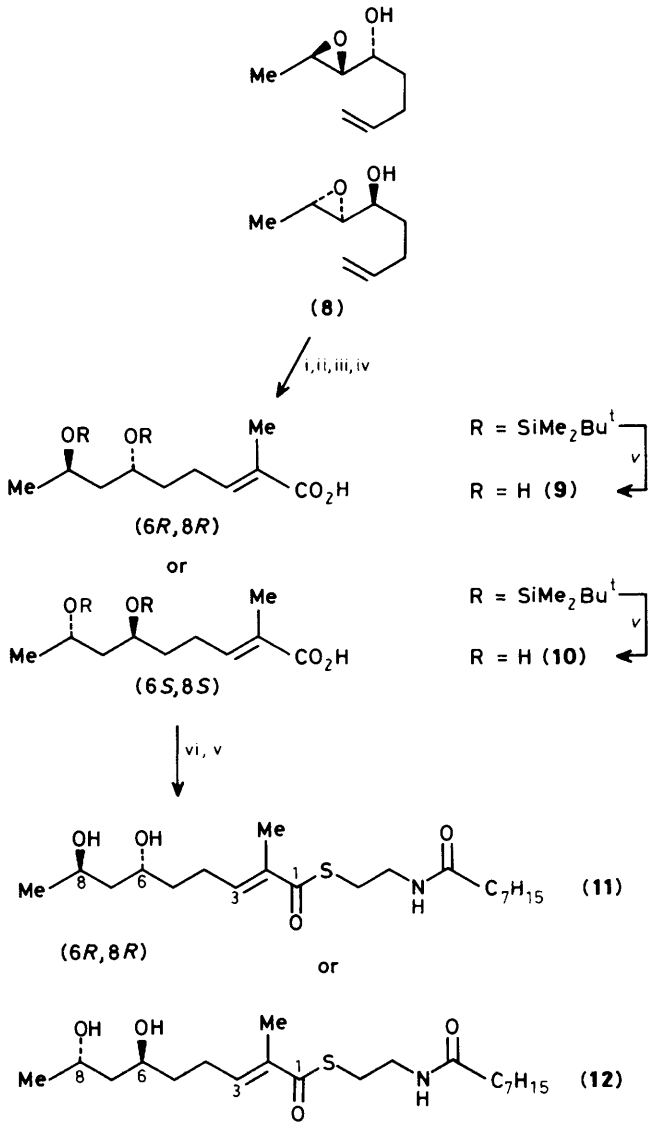
nonactin (**2**)



† All new compounds gave full spectroscopic and microanalytical data consistent with the structures shown.



Scheme 1. (SR = protein or coenzyme-A *in vivo*).

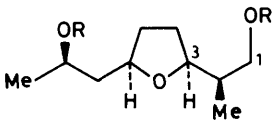


Scheme 2. *Reagents and conditions:* i, Red-Al (sodium bis(2-methoxyethoxyaluminium hydride), THF, −20 °C; ii, Bu^tMe_2SiCl, dimethylformamide, imidazole; iii, O_3, H_2–Pd work-up; iv, $(EtO)_2POC^-MeCO_2^-$; v, $BF_3{\cdot}OEt_2$, CH_2Cl_2; vi, $C_6H_{11}{\cdot}N{=}C{=}N{\cdot}C_6H_{11}$, $C_7H_{15}CO{\cdot}NH{\cdot}CH_2CH_2{\cdot}SH$.

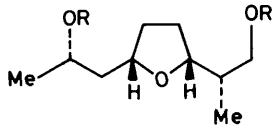
specificity of the incorporations was assayed by extracting the macrotetrolides produced during each fermentation, reducing these [$LiAlH_4$, tetrahydrofuran (THF)], derivatizing with (−)-α-methoxytrifluoromethylphenylacetyl chloride,[5] and separating the resulting diastereoisomers (**13**) and (**14**) by h.p.l.c. according to a previously published method.[3]

When the free acids (**9**) and (**10**) were tested in feeding experiments to shake cultures of *S. griseus* ETHA7796 {(6*R*, 8*R*)[1-^{14}C][1-^{13}C,3-$^2H_1$] isomer (**9**) (20 mg; 2.92 × 10^8 d.p.m./mmol) in admixture with (6*S*,8*S*)[3-^{3}H] isomer (**10**) (20 mg; 2.59 × 10^9 d.p.m./mmol) administered to a 60 ml culture} both ^{3}H and ^{14}C labels were incorporated into nonactin, but in a non-stereospecific manner; the diastereoisomers (**13**) and (**14**) each contained both ^{3}H and ^{14}C enrichments (the specific incorporations were 0.5—2.95%). It is not clear to what extent the precursors have been degraded, or otherwise modified prior to incorporation, but in addition to nonactin, small quantities of the keto-alcohol (**15**), as well as unconverted diols (**9**) and (**10**), could be extracted from the fermentation broth, indicating at least that the precursors can undergo oxidation at C-8.

In contrast to this, the thiol esters (**11**) and (**12**) in labelled form {(6*S*,8*S*)[3-^{3}H] isomer (**12**) (5 mg; 1.07 × 10^{10} d.p.m./mmol) in admixture with (6*R*,8*R*)[1-^{13}C,3-$^2H_1$] isomer (**11**) (80 mg; 90 atom % ^{13}C, 70 atom % $^2H_1$)}, when administered to two shake flask cultures, led to the recovery of macrotetrolide antibiotic, and subsequently to derivatives (**13**) and (**14**), showing a high and stereospecific incorporation of the labels. Thus only the isomer (**14**) derived from (−)-(6*S*,8*S*)-nonactic acid contained the ^{3}H label (1.18 × 10^8 d.p.m./mmol; 42% absolute incorporation and 1.1% specific incorporation), whereas when both were examined by ^{13}C and ^{2}H n.m.r. spectroscopy, only the (6*R*,8*R*)-isomer (**13**) showed enrichments in both the ^{13}C and ^{2}H n.m.r. spectra, consistent with specific incorporations of label at C(1) and H(3), respectively. In this experiment, no unreacted precursor (nor any derivative thereof) was detected after extraction of the whole fermentation broth. Two control experiments were also carried out to assess the chemical stability of the diol precursors in the fermentation broth. In these, the radiolabelled acid (**9**) and

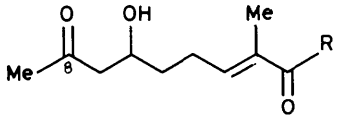


(6*R*,8*R*)-(**13**)



(6*S*,8*S*)-(**14**)

R = Ph(MeO)(CF_3)C•CO-



(**15**) R = OH

(**16**) R = $-SCH_2CH_2NHCO•C_7H_{15}$

thiol ester (**11**) were added to separate heat inactivated fermentation cultures, and incubated on a shaker at 30 °C for 6 days. In each case the precursor could be recovered in greater than 90% isolated yields, without modification.

The substantial and enantiospecific incorporations achieved using the thiol esters are noteworthy and provide compelling evidence for the involvement of these C_{10} fatty acids in the biosynthesis of the enantiomeric nonactic acids and nonactin. These results are certainly not consistent with the precursor having undergone degradation prior to incorporation, although it must be borne in mind that by a single oxidation of the diol it remains possible to generate keto-alcohol (**16**), prior to cyclisation. The present work, therefore, leads to the identification of diols (**11**) and (**12**), or possibly the adduct (**16**), as potential substrates for the enzyme catalysed cyclisation (formally a *syn*-Michael addition) needed to generate the two new chiral centres in nonactic acid. This now becomes a viable target for enzyme assays in cell-free systems, which may also be facilitated by the availability of cloned DNA encoding the nonactin biosynthetic pathway.[6]

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