Structure of Brassicicolin A: A Novel Isocyanide Antibiotic from the Phylloplane Fungus *Alternaria brassicicola*

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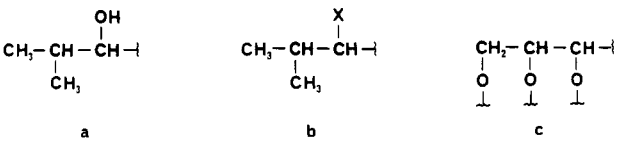
Studies of the secondary metabolites produced in culture by the plant pathogenic phylloplane fungus *Alternaria brassicicola* (Schweinitz) Wiltshire (NRRL 1299 = ATCC 6650) have led to the isolation of brassicicolin A (**6**), a novel isocyanide antibiotic with potent in vitro activity against the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*. Brassicicolin A is a fully acylated derivative of D-mannitol containing two D-α-hydroxyisovaleryl units, two acetyl groups, and two epimerized α-isocyanoisovaleryl units. The structure of brassicicolin A was assigned as **6** primarily through chemical degradation studies and by NMR and mass spectral analysis of its deisocyanation product. This account represents the first report of the α-isocyanoisovaleryl group as a naturally occurring structural unit.

Our interest in plant pathogenic and phylloplane fungi led us to examine members of the genus *Alternaria* for secondary metabolites with potentially useful biological activities. In our preliminary studies of this class of organisms, we encountered reports of antagonistic activity associated with various *Alternaria* species against competing microorganisms within the phylloplane.[1,2] In addition, there are literature reports of the production of unknown antibiotics by *Alternaria* species in liquid culture.[3,4] Lindenfelser and Ciegler proposed the name brassicicolin A for the antibiotic complex derived from *Alternaria brassicicola* (Schweinitz) Wiltshire.[4] Our efforts to follow up these reports have led to the isolation and structure determination of brassicicolin A.

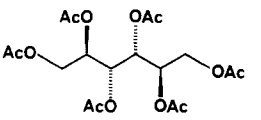
Results and Discussion

Ethyl acetate extracts of the culture filtrate and air-dried mycelia from submerged cultures of *A. brassicicola* (NRRL 1299 = ATCC 6650) were subjected to separation on silica gel and Sephadex LH-20 to afford a metabolite (brassicicolin A) with potent antibacterial activity. Analysis of brassicicolin A by FABMS with 4:1 dithiothreitol–dithioerythritol (DTT:DTE) as the matrix gave different results from those obtained with a glycerol matrix. High-resolution FABMS analysis indicated that the differences were caused by addition of one or two matrix molecules to the M + H ion when the spectrum was obtained with DTT:DTE. These results suggested the molecular formula $C_{32}H_{48}N_2O_{14}$ for brassicicolin A (m/z 685.3200 for M + H, Δ 1.8 mmu). The IR spectrum contained major absorptions at 3400, 1760, and 2150 cm^{-1}, signifying the presence of free hydroxyl, ester, and triple-bonded or cumulated double bond containing functional groups, respectively. The UV spectrum showed only end absorption. Brassicicolin A gave a negative ninhydrin test and did not react with diazomethane, thereby confirming the absence of primary and secondary amino groups and carboxylic acid functionalities. Proton and carbon-13 NMR spectral data were exceedingly complex, suggesting that the sample being analyzed was actually a mixture of very closely related compounds. The mixture was partially resolved by reversed-phase HPLC, but peak-shaving procedures afforded samples identical with the original mixture, as determined by NMR and HPLC analysis. Thus, brassicicolin A appeared to exist as an equilibrium mixture of epimers. NMR data indicated that each component of the mixture contained isopropyl, hydroxyl, acetyl, and carboxyl groups but no olefinic, aromatic, or ketone groups. Homonuclear decoupling experiments and a 2D-COSY study revealed the presence of the isolated spin systems a, b, and c, as well as acetyl groups. The molecular weight of



brassicicolin A and the large number of similar signals implied that each epimer contains two of each of these units, apparently interconnected by ester linkages. The disposition of the two nitrogen atoms could not yet be determined, though the IR adsorption at 2150 cm^{-1} suggested that at least one of these might be present as part of an isocyanide group.

In order to obtain unambiguous structural information about the individual units and to locate the site(s) of epimerization, brassicicolin A was subjected to chemical degradation experiments. Mild basic hydrolysis, followed by acetylation and solvent partitioning, afforded the hexaacetyl derivative of a symmetrical hexitol. The structure of this product was assigned as D-mannitol hexaacetate (**1**) by comparison of its spectral properties and optical rotation to those of a synthetic standard.



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Acid hydrolysis of brassicicolin A gave a single organic-soluble product which was identified as D-α-hydroxyisovaleric acid (**2**) by GC/MS, NMR analysis, and comparison with an authentic standard. A single ninhydrin-positive component was found in the water-soluble portion of the hydrolyzate. Treatment with trifluoroacetic anhydride, followed by hydrogen chloride in 1-butanol, afforded *N*-(trifluoroacetyl)valine *n*-butyl ester (**3**),[5] suggesting the

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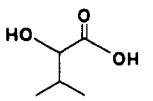

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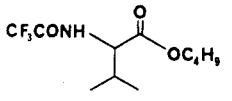

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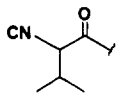
presence of valyl unit(s) in brassicicolin A. Analysis of the hydrolyzate by chiral TLC indicated that the valine obtained is racemic, implying that the site of epimerization in brassicicolin A is the α-carbon of the valine unit. The absence of amide or primary amino groups in brassicicolin A and the facility of the epimerization process indicated that the valyl unit(s) are somehow modified in the natural product. The IR band at 2150 cm^{-1} and the complex, broadened signals at 161 ppm in the carbon-13 NMR spectrum, both characteristic of isocyanide groups,[6] revealed that the valine obtained as a hydrolysis product arises from the presence of α-isocyanoisovaleryl units (**4**) in brassicicolin A. Structural unit **4** has not been previously reported as a subunit of a natural product.



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Confirmation of the presence of α-isocyanoisovaleryl units was obtained by conversion of the isocyanide functionalities into formamide groups.[7] Treatment of brassicicolin A with 1:1 methanol–acetic acid produced a product mixture which no longer gave a characteristic isocyanide IR band, but showed a broad amide absorption at 1660 cm^{-1}. The proton NMR spectrum of this product contained new formamide C–H proton signals between 8.20 and 8.25 ppm, coupled to new formamide N–H proton signals at 6.30–6.35 ppm, which were, in turn, coupled into the spin systems of the valine units.

All of these results were completely consistent with the spin systems suggested by the ^1H NMR experiments. Therefore, on the basis of the proposed molecular formula, brassicicolin A consists of two α-isocyanoisovaleryl units, two D-α-hydroxyisovaleryl units, and two acetyl units which must be involved in a total of six ester linkages with the oxygen atoms of mannitol.

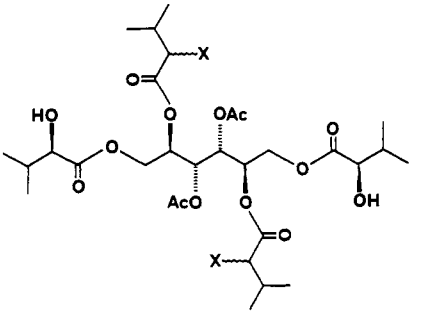
In an effort to effect a simplification of the NMR spectral data and to allow a determination of the connectivity of these units, brassicicolin A was subjected to reductive deisocyanation with tri-*n*-butyltin hydride.[8] A single product (M + H, 635.3316; $C_{30}H_{50}O_{14}$ + H) was obtained, and its NMR spectra (Table I) were dramatically simplified relative to those of the natural product. These data clearly showed that the epimeric centers had been eliminated by conversion of the α-isocyanoisovaleryl units to isovaleryl units. Furthermore, the resulting product was completely symmetrical, as indicated by the presence of half the number of NMR signals expected for a nonsymmetrical structure. This result reduced the number of possible sequences to three, and the acyl groups were correlated with nearby protons of mannitol through long-range carbon–hydrogen decoupling experiments.

Irradiation of the proton signal at 5.40 ppm (mannitol H-3) collapsed the ester carbonyl multiplet at 169.4 ppm to a sharp quartet, the irradiation of the acetyl methyl proton singlet collapsed the same carbon signal to a doublet. Thus, the acetyl units acylate the oxygens at the 3- and 4-positions of mannitol. Irradiation at each of the geminal proton signals at 4.45 and 4.07 ppm and the nearby α-hydroxyisovaleryl methine resonance at 4.02 ppm dramatically simplified the ester carbonyl signal at 174.3 ppm, indicating that the α-hydroxyisovaleryl units acylate the oxygens at the 1- and 6-positions of mannitol. By a process of elimination, the isovaleryl units must acylate the oxygens at the 2- and 5-positions of mannitol. This conclusion was confirmed by irradiation of the proton signals at 2.18 and 5.05 ppm, since each of these experiments simplified the remaining carbonyl signal at 171.8 ppm. These data permitted assignment of the structure of the deisocyanation product as **5**, and the structure of brassicicolin A was therefore assigned as **6**.

Table I. NMR Data for Deisocyanobrassicicolin A (5)[a]

position	^{13}C	^1H	corresponding ^1H NMR signals for 6[b]
		Mannitol Unit	
C-1, C-6	62.4 (t)	4.45 dd (2.4, 12.5 Hz)	4.39
		4.07 dd (4.6, 12.5)	4.05
C-2, C-5	67.7 (d)	5.05 ddd (2.4, 4.6, 8.5)	5.05
C-3, C-4	67.3 (d)	5.40 d (8.5)	5.49
		Hydroxyisovaleryl Units	
C=O	174.3 (s)		
α-CH	75.1 (d)	4.02 dd (5.9, 3.6)	4.01
OH		2.64 d (5.9)	2.68
β-CH	31.9 (d)	2.07 m[a]	2.04
γ-CH_3	18.8 (q)	1.01 d (6.9)[b]	1.12
	16.0 (q)	0.86 d (6.9)[b]	0.87
		Isovaleryl Units (Isocyanoisovaleryl Units in 6)	
C=O	171.8 (s)		
α-CH_2	42.9 (t)	2.16 m	4.05 (CHNC)
β-CH	25.3 (d)	2.05 m[a]	2.31
γ-CH_3	22.4 (q)	0.94 d (6.6)[b]	1.12
	22.4 (q)	0.93 d (6.6)[b]	0.99
		Acetyl Units	
C=O	169.4 (s)		
CH_3	20.6 (q)	2.09 s	2.11

[a] All spectra were recorded in $CDCl_3$. Chemical shifts are given in ppm downfield from TMS. Proton assignments with identical superscript letters are interchangeable (a, b). [b] Values given are approximate shifts for groups of overlapping signals.



5 X = H

6 X = NC

The facility of the epimerization in structure **6** was confirmed by deuterium-exchange and variable-temperature NMR experiments. The α-protons of the isocyanoisovaleryl units resonating at approximately 4.05 ppm were exchanged with deuterium upon standing overnight in CD_3OD–D_2O, as determined by inspection and integration of the proton NMR spectrum. In addition, when proton NMR spectra of brassicicolin A were obtained at incre-

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mentally elevated temperatures, the α-isocyanoisovaleryl α-proton signals were clearly sharpened, indicating an increase in the rate of interconversion.

The NMR data obtained for **6** was consistent with the presence of a mixture of all three possible stereoisomers. Since one of the possible isomers is nonsymmetrical, a total of four sets of similar signals should be present. Although most of the signals in the proton NMR spectrum could not be resolved, integration of the hydroxyl group doublets gave the 1:1:1:1 ratio expected for a statistically random distribution of stereoisomers. Efforts to detect the ^{14}N–^{1}H or ^{14}N–^{13}C couplings frequently observed for isocyanides[6] were unsuccessful due to the complexity of the NMR spectra.

The occurrence of racemic α-carboxyl isocyanide natural products has been previously documented,[9] and the synthetic preparation of optically active α-isocyano esters is reportedly difficult in many cases.[10] Naturally occurring isocyanides are rare, but have been reported from marine sponges,[11,12] as well as microbial sources.[9,13,14]

Brassicicolin A exhibits potent activity against Gram-positive bacteria. Standard disk assays yielded zones of 24 and 21 mm against *Staphylococcus aureus* and *Bacillus subtilis*, respectively, at 5 μg/disk. Determination of precise MIC values was hindered by lack of water solubility, but substantial reduction of growth of *S. aureus* and *B. subtilis* was observed at concentrations of 1.5 and 8.0 μg/mL, respectively. Brassicicolin A is inactive against the yeast *Candida albicans* and is nontoxic to brine shrimp at 1 mg/mL. *A. brassicicola* is pathogenic to a variety of plants of the genus *Brassica*. Recently, uncharacterized phytotoxins from *A. brassicicola* have been reported as potent inhibitors of pollen germination in various species of *Brassica* plants,[15] but it remains to be determined whether brassicicolin A is responsible for this effect.

Experimental Section

General Procedures. All solid and liquid culture media were sterilized by autoclaving at 121 °C and 15 psi for 15 min. Stock cultures were maintained on Difco potato dextrose agar (PDA) slants at 4 °C. Column chromatography employed silica gel (80–230 mesh; Baker). TLC was performed with glass plates precoated with silica gel F-254 (0.25-mm thickness, E. Merck), and TLC spots were visualized by examination under a UV lamp or by exposure to iodine vapor. Reversed-phase HPLC was carried out with a Beckman Model 332 gradient system with a Model 163 variable-wavelength UV detector and an Altex semipreparative ODS column (5-μm particles; 25 mm × 10 mm i.d.; flow rate, 2.0 mL/min; monitored at 215 nm). Proton and carbon-13 NMR spectra were recorded on a Bruker WM-360 spectrometer at 360 and 90.7 MHz, respectively. Chemical shifts are reported in ppm downfield from tetramethylsilane with the chemical shift of the residual protiated solvent as a reference. Electron-impact mass spectra were obtained at 30 eV with a Hewlett-Packard 5985B mass spectrometer with direct inlet probe. High- and low-resolution FAB mass spectra were obtained with a VG ZAB-HF double-focusing instrument equipped with an Iontech FAB gun using a beam of xenon atoms and either glycerol or 4:1 DTT:DTE as a liquid matrix. IR spectra were measured on a Beckman Acculab 1 instrument, UV spectra were recorded on a Varian-Cary 219 spectrophotometer, and optical rotations were determined on a Perkin-Elmer 141 polarimeter. Microanalyses were performed by Desert Analytics, Inc., Tucson, AZ, and authentic standards of α-hydroxyisovaleric acid, mannitol, and valine were obtained from Sigma Chemical Co.

Cultivation of *A. brassicicola*. Three isolates of *Alternaria brassicicola* (NRRL 1299, 2167, and 8052) were obtained from the Agricultural Research Service Culture Collection, USDA, Northern Regional Research Center in Peoria, IL. All three cultures were found to produce the same metabolites in liquid culture, but NRRL 1299 was chosen for this work because it produced higher titers of the compounds of interest. Two-liter Erlenmeyer flasks, each containing 450 mL of *Alternaria* production medium,[3] were inoculated with several 1-cm^2 plugs taken from 3-day-old petri dish cultures of *A. brassicicola*. Flask cultures were incubated at 25–28 °C and aerated by agitation on an orbital shaker at 200 rpm. Concentrations of the active metabolites were monitored by bioassay of the culture filtrate and/or by TLC. Antibiotic activity of the culture filtrate reached a maximum after 5–6 days.

Isolation of Brassicicolin A (6). The filtered culture broth (2700 mL) was extracted with ethyl acetate (4 × 500 mL), and the organic phase was dried ($MgSO_4$) and evaporated to afford 432 mg of a yellow oil which contained most of the antibiotic activity. The oil was chromatographed on a silica gel column (4 × 65 cm) using a stepwise gradient from 5 to 60% (v/v) ethyl acetate in hexane, with collection of 5-mL fractions. Fractions of similar composition as determined by TLC analysis were pooled and bioassayed. Fractions which showed activity were combined and purified further by chromatography on Sephadex LH-20, eluting successively with 50 mL each of 4:1 dichloromethane–hexane, 3:2 dichloromethane–acetone, and 1:4 dichloromethane–acetone,[16] while 3-mL fractions were collected. Fractions 20–55 were combined to give brassicicolin A (**6**; 26.3 mg). Analysis of brassicicolin A by reversed-phase semipreparative HPLC (C_{18}; 70:30 acetonitrile–water) gave a broad peak with two shoulders centered at a retention time of 14 min. Peak-shaving invariably afforded the same epimeric mixture upon NMR analysis and the same results upon HPLC reinjection, suggesting an equilibration process and preventing resolution of the epimers. Other HPLC phases (cyanopropyl, PRP-1, silica) gave analogous results. Brassicicolin A is a colorless oil which shows a single spot on TLC at R_f 0.42 (9:1 chloroform–methanol) and gives the following physical and spectral data: $[\alpha]_D$ +16.1° (*c* 1.86, CH_2Cl_2); UV (MeOH), end absorption; IR (neat) 3460 (OH), 2955, 2905, 2860, 2155 (NC), 1740 (C=O, br), 1456, 1367, 1254, 1210, 1140, 1100, 1072, 1035 cm^{-1}; EIMS; major ions at *m/z* 684 (M$^+$, 1.0% of base peak), 641 (6.5), 568 (3.3), 542 (5.4), 514 (9.2), 441 (9.5), 399 (8.0), 315 (21), 215 (42), 181 (52), 153 (57), 115 (100), 83 (57), 43 (36). Matrix adducts were predominant in the FAB mass spectrum obtained in DTT:DTE, but were not observed when glycerol was used. This result is consistent with the facile reaction of isocyanides with thiols.[10] FABMS (glycerol) gave major ions at *m/z* 685 (M + H, 3.8%), 576 (1.6), 558 (4.2), 533 (2.0), 467 (4.2), 449 (13), 407 (4.0), 349 (11), 289 (4.3), 249 (15), 231 (18), 189 (17), 111 (45), 83 (79); ^1H NMR ($CDCl_3$), see Table I; the ^{13}C NMR spectrum ($CDCl_3$) showed 47 resolvable signals, some of them clearly containing more than one carbon and some of them overlapping, as expected for an epimeric mixture; HRFABMS obsd *m/z* 685.3200 (M + H), calcd for $C_{32}H_{48}N_2O_{14}$ + H *m/z* 685.3182. Anal. Calcd for $C_{32}H_{48}N_2O_{14}$: C, 56.14; H, 7.07; N, 4.09. Found: C, 56.38; H, 6.86; N, 4.02.

Acid Hydrolysis of 6. A sample of brassicicolin A (16 mg) was placed in a vacuum hydrolysis tube along with 5 mL of 6 N HCl. The mixture was sealed in vacuo and heated to 110 °C for 24 h. Upon cooling, the resulting solution was extracted with dichloromethane (3 × 5 mL) and the organic phase was dried ($MgSO_4$) and evaporated to give 2.0 mg (36% yield) of D-α-hydroxyisovaleric acid (**1**), as identified by comparison with an authentic standard (^1H NMR, GC/MS, $[\alpha]_D$, mp). TLC analysis of the hydrolyzate aqueous phase (4:1:1 *n*-BuOH–HOAc–H_2O)

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showed a single ninhydrin-positive spot. A small portion of the aqueous phase was evaporated and subjected to standard derivatization procedures[17] to give the trifluoroacetyl *n*-butyl esters of any amino acids present. Analysis by GC/MS indicated the presence of *N*-(trifluoroacetyl)valine *n*-butyl ester (**2**). TLC analysis of the underivatized hydrolyzate on Chiralplates (Macherey-Nagel) and comparison with authentic standards indicated that the valine obtained from hydrolysis of brassicicolin A was racemic.

Basic Hydrolysis of 6. A sample of brassicicolin A (10 mg) was dissolved in 3 mL of methanol to which 10 mg of Na_2CO_3 was added. The mixture was allowed to stir at room temperature for 24 h and was then evaporated, dissolved in 3 mL of water, and extracted with dichloromethane (3 × 5 mL). The aqueous phase was collected and evaporated to give a white residue, which was taken on to the next step without further purification.

Acetylation of Basic Hydrolysis Product. The residue obtained after basic hydrolysis as described above was suspended in 3 mL of pyridine to which 0.5 mL of acetic anhydride was added. The mixture was allowed to stir for 18 h and was subsequently evaporated, dissolved in water, and extracted with dichloromethane (3 × 5 mL). The organic phase was dried ($MgSO_4$) and evaporated to afford 4.2 mg (67% yield) of hexaacetyl-D-mannitol (**3**). The structure and stereochemistry of this product were assigned by comparison to an authentic standard prepared by acetylation of D-mannitol under identical conditions (GC/MS, HRMS, ^{1}H NMR, $[\alpha]_D$).

Selective Hydrolysis of the Isocyanide Groups. A small amount (2.0 mg) of brassicicolin A was dissolved in 1 mL of methanol. Three drops of acetic acid were added, and the solution was allowed to stand at room temperature overnight. Evaporation of the solvent and analysis by IR and ^{1}H NMR demonstrated the absence of isocyanide groups (no IR band at 2150 cm^{-1}; no C*H*NC proton NMR multiplet at 4.05 ppm), and the presence of formamide groups [broad new IR absorption at 1660 cm^{-1}; new ^{1}H NMR signals at 6.30–6.35 (N*H*CHO), 8.20–8.25 (NHC*H*O), and 4.60–4.65 ppm (C*H*NHCHO)].

Reductive Deisocyanation of 6. A sample of brassicicolin A (38 mg, 0.056 mmol) and a catalytic amount of AIBN (0.1 mg) were dissolved in 15 mL of anhydrous benzene under a nitrogen atmosphere, and 32.6 mg (0.112 mmol) of tri-*n*-butyltin hydride was added via syringe.[8] After stirring for 8 h at 80 °C, the solution was cooled, evaporated to dryness, and chromatographed on a small column of silica gel (1 × 5 cm) using a stepwise gradient from hexane to ethyl acetate. Fractions collected at 20% ethyl acetate were pooled to give 11 mg (31% isolated yield) of the white crystalline symmetrical reduction product **5**. Compound **5** has the following properties: R_f 0.71 (9:1 chloroform–methanol); mp 50–51 °C; $[\alpha]_D$ +23.0° (*c* 1.10, CH_2Cl_2); IR (neat) 3517, 2962, 2934, 2875, 1743 (br), 1468, 1371, 1293, 1210, 1032, 983 cm^{-1}; EIMS (30 eV) major ions at *m/z* 634 (M^+, 2.1), 574 (19), 518 (32), 475 (23), 417 (100), 358 (12), 318 (12), 299 (12), 257 (35), 185 (49), 157 (35), 115 (13), 85 (39), 57 (9.7); FABMS (thioglycerol) major ions at *m/z* 635 (M + H, 7.8%), 617 (5.9), 575 (18), 533 (5.3), 517 (16), 475 (16), 428 (34), 417 (3.4), 333 (3.5), 291 (4.7), 85 (42), 57 (40); ^{1}H NMR ($CDCl_3$), see Table I; ^{13}C NMR ($CDCl_3$), see Table I; HRFABMS obsd 635.3316, calcd for $C_{30}H_{50}O_{14}$ + H 635.3278. Anal. Calcd for $C_{30}H_{50}O_{14}$: C, 56.78; H, 7.94. Found: C, 56.78; H, 7.65.

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Conversion of Dehydroabietic Acid into 20-Keto-*C*-aryl-18-norsteroids. Formation of the D Ring

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Dehydroabietic acid (**1a**) has been converted into 17-epimeric 20-keto-*C*-aryl-18-norsteroids **13** via a sequence of trasformations involving as key step the regioselective functionalization of the 13-isopropyl group of **1a**.

The preparation of ring C aromatic steroids has attracted the attention of many workers due to their interesting pharmacological properties. In recent years several *C*-aryl-18-norsteroids have been totally synthesized[1] or prepared from different resin acids[2] and by conversion of naturally ocurring steroids to the C-aromatic system.[3]

In this paper we describe the elaboration of the five-membered D ring of a 20-keto-*C*-aryl-18-norsteroid from readily available dehydroabietic acid (**1a**), which with its aromatic ring C and C-13 isopropyl side chain has shown to be a suitable starting material for the preparation of 15- and 17-keto-18-norsteroids.[4a,b] The synthetic route used to prepare this ring C-aromatic steroid system is outlined in Schemes I and II.

The main synthetic problem associated with the use of this resin acid as the starting material for the preparation of C-aromatic steroids is the regioselective functionalization of the C-13 isopropyl group; the use of oxidizing agents such as CrO_3,[5a] $KMnO_4$,[5b] SeO_2,[5c] or NBS[5d] results in the oxidation of both activated benzylic positions or in the

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