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Cultures of Separated Mating Types of *Blakeslea trispora* Make D and E Forms of Trisporic Acids†

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ABSTRACT: Trisporic acids are end products of the sex-specific pheromones in mucoraceous fungi. We have found three new trisporic acids in cultures of *Blakeslea trispora* in which (+) and (−) mating types were separated by a membrane with 0.45-μm pores. Two of the trisporic acids were new compounds; the structure of the third [previously described by Miller and Sutter [(1984) *J. Biol. Chem. 259*, 6420] as methyl trisporate-E with a hydroxyl group at C-2] was revised. Trisporic acid-E(3*R*), trisporic acid-E(3*S*), and trisporic acid-D(2*S*) were in a 1:1:2 ratio, accounted for 9% of the total trisporic acids, and differed by the position and configuration of a hydroxyl group on the ring at C-2 or C-3, the conformation of the ring, the extent of rotation of the side chain relative to the ring, and either a carbonyl or hydroxyl group on the side chain at C-13. These three compounds accounted for only 0.5% of the total trisporic acids in combined mating type cultures. Since the combined cultures did not metabolize trisporic acid-E(3*R*), its biosynthesis apparently ceases when opposing mating types contact each other physically. We speculate that *B. trispora* and *Phycomyces blakesleeanus* utilize different pheromones to regulate an early event (possibly zygotropism) in sexual development.

Trisporic acids are oxygenated, 18 carbon atom derivatives of β-carotene (Figure 1; Caglioti et al., 1966). Trisporic acids normally are found in the medium of combined, but not separate, (+) and (−) mating type cultures of mucoraceous fungi (van den Ende, 1978; Jones et al., 1981; Sutter, 1987). Trisporic acids stimulate carotenogenesis in *Blakeslea trispora* and the development of zygophores (sexual hyphae) in *Mucor mucedo* (Caglioti et al., 1966; van den Ende, 1968). However, physiological concentrations of (extracellular) trisporic acids stimulate neither carotenogenesis nor the development of zygophores in *Phycomyces blakesleeanus* nor the development

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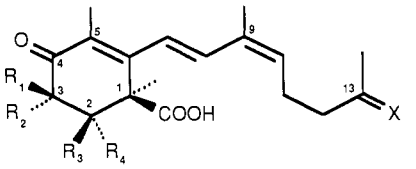

FIGURE 1: Structural formula for trisporic acids. Trisporic acid-B: $R_1 = R_2 = R_3 = R_4 = H$; X = O. Trisporic acid-C: $R_1 = R_2 = R_3 = R_4 = H$; X = H, OH. Trisporic acid-E(3*R*): $R_1 = OH$; $R_2 = R_3 = R_4 = H$; X = H, OH. Trisporic acid-E(3*S*): $R_2 = OH$; $R_1 = R_3 = R_4 = H$; X = H, OH. Trisporic acid-D(2*S*): $R_3 = OH$; $R_1 = R_2 = R_4 = H$; X = O. The designations (3*R*), (3*S*), and (2*S*) in the E and D forms of trisporic acids indicate the position and configuration of the ring hydroxyl group. Methyl ester derivatives of trisporic acids were used for the analyses described in this paper.

of zygophores in *B. trispora* (Sutter, 1987; Drinkard et al., 1982). More importantly, trisporic acids are end products of the pheromones that stimulate both carotenogenesis and the development of zygophores at least 100 more effectively in cultures of the opposite sex (van den Ende, 1978; Sutter, 1987). These sex-specific pheromones are, depending upon the species, between 100- and 100 000 000-fold less abundant than the trisporic acids. The chemical structures of eight sex-specific pheromones have been reported (Bu'Lock et al., 1974; Nieuwenhuis & van den Ende, 1975; Sutter & Whitaker, 1981). None has a hydroxyl group at C-2 or C-3; half have a hydroxyl group on the side chain at C-13, half a carbonyl group. Four sex-specific pheromones were isolated from (+) cultures of *B. trispora*, four from (−) cultures. Figure 2 illustrates the relationship between the sex-specific pheromones and trisporic acids.

Since sexual development presumably proceeds by similar mechanisms in all species of mucorales (Burgeff, 1924; Blakeslee & Cartledge, 1927; van den Ende, 1978; Jones et al., 1981; Sutter, 1987), one might expect cultures of different mucoraceous fungi to accumulate the same trisporic acids. However, that apparently is not true. Combined mating type cultures of *B. trispora* accumulate 2% trisporic acid-A, 15% trisporic acid-B, and 83% trisporic acid-C (Caglioti et al., 1966). In contrast, trisporic acid-E represents 30% of the trisporic acids made by combined mating type cultures of *P. blakesleeanus* (Miller & Sutter, 1984). Could trisporic acid-E be made by cultures of *B. trispora* in which (+) and (−) mating types are separated by a membrane? Possible support for this idea is found in a paper 15 years ago that stated that 35% of the trisporic acids made by (+) cultures of *B. trispora* incubated with extracts from (−) cultures migrated slower upon silica gel chromatography than trisporic acid-C (Sutter et al., 1974).

The first objective of the study was to determine whether membrane-separated (+) and (−) cultures of *B. trispora* made trisporic acid-E. The second objective was to determine if the virtual absence of trisporic acid-E in combined mating type cultures resulted from its being metabolized. Our results indicated that trisporic acid-E is made but is not metabolized significantly. To our surprise, membrane-separated cultures of *B. trispora* made a second isomer of trisporic acid-E and also trisporic acid-D. Spectroscopic studies partially characterizing the constitution, configuration, and conformation of these three compounds are described also.

Experimental Procedures

Membrane-separated (+) and (−) cultures and control combined (+) and (−) cultures of *B. trispora* [i.e., one culture of NRRL2895(+) per four of NRRL9159(−)] were grown for 6 days as described by Sutter and Jelinek (1983). Typically,

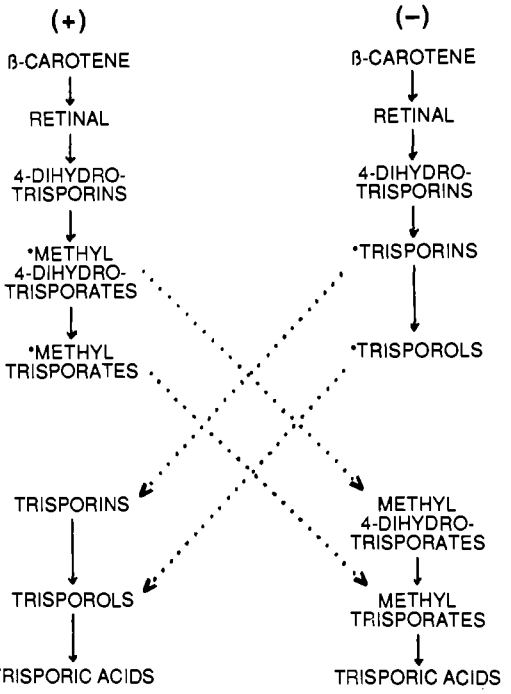

FIGURE 2: Metabolism of the sex-specific pheromones. The dots indicate the sex-specific pheromones. The solid lines represent one or more enzymatic reactions. The dotted lines represent the diffusion of compounds between mating types. Once formed, trisporic acids are released into the medium, where they accumulate. The distinguishing feature of (+) and (−) pheromones is the degree of modification of the *pro-S* methyl group on C-1 of 4-dihydrotrisporin, a putative precursor common to both mating types. The methyl group is either unaltered (e.g., trisporins) or oxidized to a primary alcohol (e.g., trisporols) in (−) pheromones and oxidized to a carboxyl group that is esterified in (+) pheromones.

prepared combined mating type cultures were inoculated at zero time with equal amounts of (+) and (−) mycelium (Sutter & Whitaker, 1981). Except for high-pressure liquid chromatography, NMR,[1] and mass spectrometry, the techniques used in the purification and analysis of trisporic acids were described by Sutter and Whitaker (1981). Trisporic acids were extracted from culture medium with chloroform and were purified by bicarbonate extraction and DEAE-Sephadex chromatography prior to analyses. Trisporic acids were esterified and resolved partially by LH-20 Sephadex chromatography with ethyl acetate. Component C, which contains the methyl esters of all three trisporic acids characterized in this paper, was resolved further by high-pressure liquid chromatography on a Porasil column (7.8 mm diameter × 30 cm) using 0.625% methanol in chloroform, 4 mL/min. Authentic trisporic acid-E was isolated from combined mating type cultures of *P. blakesleeanus* and was purified free of other trisporic acids as described by Miller and Sutter (1984). Authentic trisporic acid-E, in experiments in which it had been added to combined mating type cultures and then recovered, was resolved from the B and C forms of purified trisporic acids by silica gel thin-layer chromatography with chloroform containing 5% acetic acid.

NMR spectra of samples in deuterated chloroform were obtained by using the 620-MHz spectrometer at the NMR Facility for Biomedical Studies, Pittsburgh, by either FT or rapid scan correlation techniques (Dadok & Sprecher, 1974).

[1] Abbreviations: CI, chemical ionization; EI, electron ionization; ^2Js, geminal two-bond HH coupling constants; ^3Js, vicinal three-bond HH coupling constants; *m/e*, mass/charge; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; SSD, spin–spin decoupling.

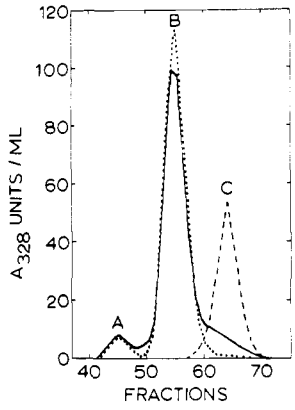

FIGURE 3: Sephadex LH-20 chromatography with ethyl acetate of methyl trisporate-E(3*R*) (dashed curve) and of methyl trisporates isolated as acids from membrane-separated (solid curve) and combined (dotted curve) (+) and (−) cultures. The absorbance values shown for the three curves are 1.5, 1.0, and 0.5 times, respectively, the actual values obtained. Component C included fractions 57–67. Each fraction was 5 mL.

Mass spectra were obtained with a Finnigan Model 4500 mass spectrometer interfaced with an Incos data system. Ten micrograms of sample in 2 μL of ethanol was placed in a glass sample holder attached to a probe. Ground glass was laid over the sample to keep it from being sucked out of the holder instantaneously by the vacuum. The probe, when placed into the spectrometer, was initially at 37 °C. The temperature was raised 30 °C/min to 250 °C. Ionization was either by electron impact at 70 eV or chemically by methane. The latter procedure adds 1 mass unit to each ion. Ions between 200 and 340 mass units were scanned every second. The following data are from the peak of the ion curves: fractions 1 and 2 (Figure 4) EI, *m/e* (rel intensity) 320 (100%, molecular ion), CI, *m/e* (rel intensity) 321 (100%, molecular ion + H); fraction 4 (Figure 4) EI scan 295, *m/e* (rel intensity) 336 (100%, molecular ion), 318 (34%), 303 (5%), 290 (10%), 275 (17%), 259 (48%), 236 (74%), 213 (41%), CI scan 124, *m/e* (rel intensity) 337 (93%, molecular ion + H), 319 (100%); fraction 5 (Figure 4) EI scan 308, *m/e* (rel intensity) 334 (92%, molecular ion), 316 (3%), 306 (19%), 284 (5%), 273 (19%), 259 (100%), 241 (34%), 213 (54%), 200 (45%), CI scan 330, *m/e* (rel intensity) 335 (100%, molecular ion + H), 317 (52%), 285 (90%).

RESULTS

Membrane-separated (+) and (−) cultures of *B. trispora* accumulated 21 ± 7 (mean, SD, 10 experiments) mg of trisporic acids/L of medium. The methyl esters of trisporic acids were resolved by Sephadex LH-20 chromatography with ethyl acetate into three components consisting mainly of methyl trisporate-B, methyl trisporate-C, and component C, which elutes with authentic methyl trisporate-E (Figure 3, parts A–C, respectively). Component C, representing 19.5% of the total methyl trisporates, was resolved into seven fractions by high-pressure liquid chromatography (Figure 4). Fractions 1, 2, 4, and 5 represented 90% of the applied sample or 2.4%, 4.9%, 5.9%, and 4.4%, respectively, of the total methyl trisporates isolated as trisporic acids from the cultures. All four fractions exhibited ultraviolet absorbance maxima in ethanol around 328 nm, indicating the chromophore contains three double bonds conjugated with a carbonyl group and is perturbed by a methoxycarbonyl group at carbon atom 1 (Sutter, 1987). Mass spectroscopy revealed that the molecular ion in fractions 1 and 2, 4, and 5 was 320, 336, and 334 mass units, respectively. These molecular masses are consistent with the molecular formulas $C_{19}H_{28}O_4$, $C_{19}H_{28}O_5$, and $C_{19}H_{26}O_5$, which

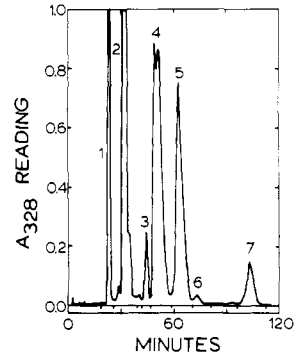

FIGURE 4: Resolution of 1.47 mg of component C (Figure 3) from membrane-separated (+) and (−) cultures by silica gel high-pressure liquid chromatography.

are characteristic of methyl trisporate-C, methyl trisporate-E, and methyl trisporate-D, respectively.

Fraction 4 contains two major and two minor compounds in a ratio of 4:4:1:1 as judged by the OCH_3 signals seen by high-resolution NMR spectroscopy. By the same criterion, fraction 5 contains a single compound. Table I presents a summary of some of the NMR data obtained with the two major compounds in fraction 4 [methyl trisporate-E(3*R*) and methyl trisporate-E(3*S*)] and with fraction 5 [methyl trisporate-D(2*S*)]. Signals were identified for all 26 hydrogens attached to carbon atoms in each form of methyl trisporate-E and for all 25 in methyl trisporate-D. The assignment of signals to specific hydrogen atoms is based upon the positions (chemical shifts) and shapes of the signals, integration curves of the signals, coupling constants between signals, and SSD and NOE experiments. For example, the chemical shift and shape of the signal for the methine hydrogen on C-10 is easy to identify in all trisporic acids because no other signal is nearby. The identity of the signals for hydrogens on C-11–C-13 in all three compounds was determined unequivocally through SSD experiments beginning at 10-H (data not shown). The signal for the methyl hydrogens attached to C-13 in the two forms of methyl trisporate-E is a doublet at 1.23 and 1.21 ppm, respectively. The doublet indicates that C-13 bears a single hydrogen. The chemical shift of this methine hydrogen indicates that a hydroxyl group also is attached to C-13 in each form of methyl trisporate-E. In contrast, the signal for the methyl hydrogens attached to C-13 in methyl trisporate-D is a singlet at 2.15 ppm, which indicates that C-13 is a carbonyl group.

The *ring* hydrogens in all three compounds are on adjacent carbon atoms as judged by SSD experiments. The ^2Js between methylene hydrogens on the ring portion of the molecule are 13.3, 13.8, and 16.9 Hz in the two forms of methyl trisporate-E and in methyl trisporate-D, respectively. These ^2Js indicate that the methylene hydrogens are on a carbon atom adjacent to saturated carbon atoms in both forms of methyl trisporate-E and adjacent to a keto group in methyl trisporate-D.[2] ^3Js indicate that the ring methine hydrogen is on a carbon atom adjacent to that with the methylene hydrogens. The chemical shift of the ring methine hydrogen indicates that a hydroxyl group is attached to that same carbon atom. Since the valencies of carbon atoms 1, 4, 5, and 6 in the ring portion of the molecule are accounted for, the hydroxyl group is on C-3

[2] The ^2Js between methylene hydrogens in a six-membered ring structure lie between 15.8 and 18.5 Hz (mean = 17.3, 6 examples) if they are on a carbon atom adjacent to a keto group and between 12.2 and 14.9 Hz (mean = 13.1, 16 examples) if adjacent to saturated carbon atoms (Bothner-By, 1965).

Table I: Assignments of 620-MHz Proton NMR Spectra of Methyl Trisporate-E(3*R*), Methyl Trisporate-E(3*S*), and Methyl Trisporate-D(2*S*)[a]

	methyl hydrogen signals					methine hydrogen signals				methylene hydrogen signals		
compd	1-OCH$_3$	1-CH$_3$	5-CH$_3$	9-CH$_3$	13-CH$_3$	7-H	8-H	10-H	13-H	ring	ring	11-H$_2$	12-H$_2$
mTA-E(3*R*)	3.670	1.698	2.036	1.870	1.231	6.380	6.923	5.683	3.80	4.323	2.321	2.283	2.21, 2.35	1.48, 1.55
	s	d (0.6)	s	s	d (6.33)	d (16.5)	d (16.5)	t (7.5)	m	dd, (6.0, 13.3)	m (6.0, 12.4)	m (0.6, 12.4, 13.3)	m, m	m, m
mTA-E(3*S*)	3.760	1.487	2.012	1.878	1.208	6.284	6.786	5.619	3.80	4.417	2.613	1.860	2.26	1.52
	s	s	s	s	d (6.15)	d (16.5)	d (16.5)	t (7.5)	m	dd (5.8, 13.8)	dd (5.8, 13.4)	m (13.4, 13.8)	m	m
mTA-D(2*S*)	3.778	1.598	1.949	1.866	2.151	6.339	6.729	5.498	none	4.032	2.814	2.796	2.421	2.527
	s	s	s	s	s	d (16.4)	d (16.4)	t (7.7)		m (3.8, 9.5)	m (9.5, 16.9)	m (3.8, 16.9)	m	t (7.1)

[a] Methyl trisporate is abbreviated mTA. The chemical shift for each signal is expressed in parts per million relative to tetramethylsilane, an internal standard. The signal shape [doublet (d), doublet of doublet (dd), multiplet (m), singlet (s), and triplet (t)] and the coupling constants (expressed in hertz) are given under the chemical shift. The chemical shifts for mTA-E(3*R*) have been reported (Miller & Sutter, 1984). SSD experiments show long-range coupling between the following pairs of signals: 12-H$_2$ to 13-CH$_3$ in mTA-D(2*S*); 10-H to 9-CH$_3$ in all three compounds; 7-H to 5-CH$_3$ in mTA-D(2*S*) and mTA-E(3*S*); and 2.28 ppm to 1-CH$_3$ in mTA-E(3*R*). NOE was seen at 10-H and 7-H in all three compounds when the signal for 9-CH$_3$ was irradiated; at 1-CH$_3$ and 11-H$_2$ in all compounds [and at 5-CH$_3$ in mTA-D(2*S*) and in mTA-E(3*S*)] when the signal for 8-H was irradiated; at 5-CH$_3$ and 9-CH$_3$ in all compounds [and very weakly at 1-CH$_3$ in mTA-D(2*S*) and mTA-E(3*S*)] when the signal for 7-H was irradiated; at 8-H in all compounds [at 7-H in mTA-D(2*S*) and in mTA-E(3*S*)] and on the ring hydrogens at 4.323 and 2.32 ppm in mTA-E(3*R*), at 2.612 and 1.86 ppm in mTA-E(3*S*), and at 4.032 and 2.796 ppm in mTA-D(2*S*) when the signal for 1-CH$_3$ was irradiated.

in both forms of methyl trisporate-E and on C-2 in methyl trisporate-D: more appropriately called methyl trisporate-E(3) and methyl trisporate-D(2), respectively.

Table II indicates how the two configurations for the hydroxyl group on either C-2 or C-3 can be differentiated. To make *R* and *S* assignments, we had to make an assumption about the configuration at C-1 because only relative configurations can be determined by NMR. It is assumed that the three trisporic acids characterized in this study have the same configuration at C-1 as (1*S*)-methyl trisporate-C (Bu'Lock et al., 1970). First, consider the two forms of methyl trisporate-E(3). In each compound the signal for the methine hydrogen shows small and large ^3Js to the methylene hydrogens.[3] When the methyl group on C-1 was irradiated, NOE was detected in the ring at the methine hydrogen and at the equatorial methylene hydrogen in one isomer of methyl trisporate-E(3) and at both methylene hydrogens in the second isomer. These observations demonstrate that models (3*R*), OH = e, and (3*S*), OH = e, in Table II represent the configurations and conformations of the first and second isomers of methyl trisporate-E(3): hereafter called methyl trisporate-E(3*R*) and methyl trisporate-E(3*S*), respectively (Figure 5, parts E and H). The four-bond w-coupling in methyl trisporate-E(3*R*) between the methyl group on C-1 and the axial methylene hydrogen at 2.28 ppm on C-2 provided additional support for these two structures and conformations. Such coupling across four single bonds without any intervening sp^2 hybridized carbon atoms is possible only if the two groups are trans to each other (Sternhell, 1964). Since the hydroxyl group is in an equatorial position in both compounds, each may maintain a single conformation through hydrogen bonding between the hydrogen of the hydroxyl group and the oxygen of the keto group to form a stable five-membered ring in chloroform.

In contrast, methyl trisporate-D(2) is in rapid equilibrium between two conformers. One of the ^3Js of the ring methine hydrogen is small; the other intermediate is between small and large.[3] These ^3Js indicate the compound is in one conformation approximately three-fourths of the time, in the second conformation the remaining time. They also indicate that either model (2*R*), OH = e, or model (2*S*), OH = e, in Table II represents the configuration of C-2 in the major conformer because these two models permit ^3Js that are greater than 4 Hz. Since the methine hydrogen exhibits an NOE when the methyl group on carbon atom 1 is irradiated, model (2*S*), OH = e, must represent the major conformer and model (2*S*), OH = a, the minor conformer of methyl trisporate-D(2): hereafter called methyl trisporate-D(2*S*) (Figure 5, parts D and C). Presumably, neither conformation is stabilized since hydrogen bonding apparently is possible in both conformations between the hydroxyl group on C-2 and the methoxycarbonyl group on C-1.

The double bonds in the side chain of all three compounds are 7*E*,9*Z* (Figure 1). The major conformation of the side chain through C-10 with respect to the ring also is shown in Figure 1. These interpretations are based upon the NOE studies described qualitatively in the legend to Table I. The

[3] The ^3Js between a methine hydrogen and the hydrogens of an adjacent methylene group in a six-membered ring structure with a chair conformation range between 2.7 and 5.4 Hz (mean = 4.0, 12 examples) if the coupled hydrogens are in a gauche relationship and between 10.5 and 12.4 Hz (mean = 11.2, 6 examples) if the hydrogens are trans diaxial (Bothner-By, 1965). ^3Js of intermediate size indicate a rapidly interconverting mixture of conformers. Our data from the two forms of methyl trisporate-E(3) indicate that the ^3Js are about 2 Hz larger when the methine hydrogen is on a carbon atom adjacent to a keto group.

Table II: Basis for Deciding the Ring Structure of the D and E Forms of Methyl Trisporates[a]

model	Figure 5	1-CH_3	OH and H on carbon atom				methine *J* with				irr 1-CH_3: NOE seen at			
			2-e	2-a	3-e	3-a	2-H(e)	2-H(a)	3-H(e)	3-H(a)	2-H(e)	2-H(a)	3-H(e)	3-H(a)
(2*R*), OH = e	A	a′	OH	H	H	H	–	●	S	L	–	N	N	Y
(2*R*), OH = a	B	e′	H	OH	H	H	●	–	S	S	Y	–	N	N
(2*S*), OH = e	D	e′	OH	H	H	H	–	●	S	L	–	Y	N	N
(2*S*), OH = a	C	a′	H	OH	H	H	●	–	S	S	Y	–	N	Y
(3*R*), OH = e	E	a′	H	H	OH	H	S	L	–	●	Y	N	–	Y
(3*R*), OH = a	F	e′	H	H	H	OH	S	S	●	–	Y	Y	N	–
(3*S*), OH = e	H	e′	H	H	OH	H	S	L	–	●	Y	Y	–	N
(3*S*), OH = a	G	a′	H	H	H	OH	S	S	●	–	Y	N	N	–

[a] Abbreviations: a, axial; a′, pseudoaxial; e, equatorial; e′, pseudoequatorial; irr, irradiate; S, small; L, large; Y, yes, N, no. Symbols: –, nonexistent; ●, methine. There are four possible configurations for the hydroxyl group on the ring at C-2 and C-3 in the D and E forms of trisporic acids. In addition, the cyclohexene ring can exist in a number of conformations. The most likely conformations are those described as a half-chair in which axial groups are staggered (Hanack, 1965). For any one cyclohexene structure, there are two half-chair conformations. Assuming the half-chair possibilities and assuming the configuration at C-1 is the same as in (1*S*)-methyl trisporate-C (Bu'Lock et al., 1970), two NMR observations will provide the configuration at the hydroxyl group and either the conformation of the ring or the proportions of the two conformers. The *J* of the methine hydrogen to a methylene hydrogen is small if the angle between the atoms is 60° (gauche) or large if the angle is 180° (trans) (Bothner-By, 1965). Molecular models reveal that the methyl group on C-1 will be near one or two of the hydrogens on C-2 and C-3. These hydrogens are identifiable by NOE when the methyl group is irradiated.

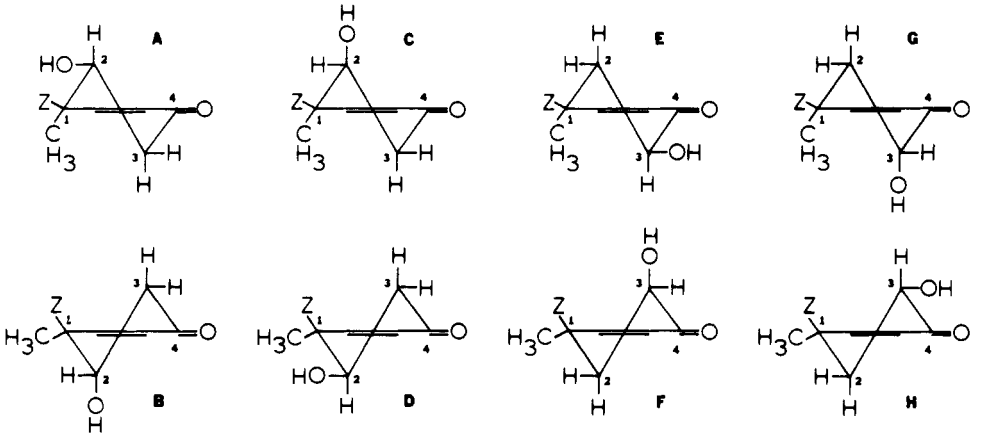

FIGURE 5: Projections of the models described in Table II. Z = $COOCH_3$; the substituents on C-5 and C-6 are not shown. The top line depicts the configurations in the half-chair conformation in which 1-CH_3 is pseudoaxial; the bottom line depicts the same configurations in the half-chair conformation in which 1-CH_3 is pseudoequatorial. Interchangeable conformations are A and B, C and D, E and F, and G and H.

quantitative studies implied the side chain rotates very little about the single bond between C-6 and C-7 in methyl trisporate-E(3*R*), rotates intermediate amounts in methyl trisporate-E(3*S*), and rotates freely in methyl trisporate-D(2*S*). We speculate that rotation about the bond between C-6 and C-7 is enhanced by changes in ring conformation and is impeded when the methoxycarbonyl group on C-1 is in a pseudoequatorial position.

Combined (+) and (–) cultures of *B. trispora* accumulated 78 mg of trisporic acids/L of medium. Component C was analyzed by silica gel high-pressure liquid chromatography. Together the E and D forms of methyl trisporates represent 0.8% and 0.5% of the total trisporic acids isolated from control and typically prepared combined (+) and (–) cultures, respectively.

Trisporic acid-E(3*R*) (3.2 mg) was added to a 48-h typically prepared combined (+) and (–) culture and to 100 mL of uninoculated medium. After the flasks were incubated on a shaker for an additional 48 h, the medium was collected. Ten milligrams of trisporic acid-C was added as carrier to the uninoculated medium prior to extraction and analysis. About 50% of the total trisporic acids were recovered in each of three experiments; the mean yield ± SD of trisporic acid-E(3*R*) recovered from the culture and uninoculated medium was 1.6 ± 0.1 and 1.4 ± 0.1 mg, respectively. Thus, trisporic acid-E(3*R*) apparently is not metabolized extensively by combined mating type cultures. This implies that the biosynthesis of trisporic acid-E(3*R*) ceases when cultures of the opposite sex make physical contact.

Discussion

We have demonstrated that membrane-separated and combined mating type cultures of *B. trispora* accumulate different ratios of trisporic acids. Membrane-separated cultures accumulated three forms of trisporic acids not reported previously in *B. trispora*. The structures of these three trisporic acids were determined primarily through high-resolution NMR spectroscopy. We began our investigation by assuming all three compounds were trisporic acids. At the end, all NMR spectra were consistent with the proposed structures. Every one of the expected hydrogen atoms was identified. Supporting evidence came from three sources: (a) the purification properties of the three compounds; (b) UV spectroscopy that revealed the conjugated system; and (c) low-resolution mass spectroscopy that provided molecular weights. Trisporic acid-E(3*R*), trisporic acid-E(3*S*), and trisporic acid-D(2*S*) were in a 1:1:2 ratio, accounted for 9% of the total trisporic acids,[4] and differed by the position and configuration of a hydroxyl group on the ring at C-2 or C-3, the conformation

[4] The D and E forms of trisporic acids accounted for 25% of the total trisporic acids in membrane-separated cultures of NRRL9246(+) and NRRL9159(–). Unfortunately, NRRL9246(+) died before the structural studies described in this paper were initiated.

of the ring, the extent of the rotation of the side chain relative to the ring, and either a carbonyl or hydroxyl group on the side chain at C-13 (Figure 1). These three acids represented only 0.5% of the total trisporic acids in combined mating type cultures.

The D and E forms of trisporic acids have the same functional group on the side chain at C-13 as the B and C forms, respectively, but in addition have a hydroxyl group on the ring portion of the molecule at either C-2 or C-3 (Figure 1). Since 9*E* isomers of B and C forms of trisporic acids also are known (Sutter, 1987), it is possible that eight isomers of trisporic acid-D and eight isomers of trisporic acid-E exist. DeHaven (1973) had found that extracts of *P. blakesleeanus* contained two trisporic acids not reported previously. The new trisporic acids migrated below trisporic acid-C upon silica gel thin-layer chromatography and were called trisporic acid-D and trisporic acid-E on the basis of their polarities.[5] Interestingly, trisporic acid-D(2*S*) is more polar upon silica gel high-pressure liquid chromatography than one would predict on the basis of its functional groups (Figure 4). A possible explanation for this behavior may be its conformation. In one conformer of methyl trisporate-D(2*S*), the ring hydroxyl group is axial, which may allow tighter binding to silica gel than an equatorial hydroxyl group. Since trisporic acid-D(2*S*) and trisporic acid-E(3*S*) migrate with trisporic acid-E(3*R*) upon silica gel thin-layer chromatography, trisporic acid-D(2*S*) and trisporic acid-E(3*S*) are new compounds not reported previously.

Trisporic acid-E(3*R*) was reported in combined mating type cultures of *P. blakesleeanus* (Miller & Sutter, 1984). The assignment of the ring hydroxyl group's position was in error because the NOE data was misinterpreted even though all NMR data reported for methyl trisporate-E(3*R*) are correct. Furthermore, the ring hydroxyl group's position in apotrisporin-E was assigned correctly, but for the wrong reason (Sutter, 1986). Two valid reasons for the assignment follow. First, the coupling constant of 17 Hz between the ring methylene hydrogens indicates they are attached to a carbon atom adjacent to a keto group.[3] This means these hydrogens are on C-3 and that the ring methine hydrogen and hydroxyl group are on C-2. Second, NOE was detected at the signals for the ring methine hydrogen and for one ring methylene hydrogen, a different one for each methyl group, when the methyl groups on C-1 were irradiated separately. These NOE observations are possible only if the hydroxyl group and methine hydrogen are on C-2. Apotrisporin-E should be renamed apotrisporin-E(2) since more than one isomer may exist.

With the discovery of D and E forms of trisporic acids, it seems likely that the corresponding forms of sex-specific pheromones exist. If it is assumed that these pheromones do exist, it is likely that *B. trispora* and *P. blakesleeanus* utilize different pheromones to regulate an early event in sexual development since the former makes two trisporic acids that the latter does not make or at least makes in a greatly different ratio. What is this early event in sexual development? It cannot be the stimulation of carotenogenesis or the development of zygophores since the same compounds are active in both species. The early event could be zygotropism: the directed growth of zygophores to their mates. We speculate that the mating type specific precursors of any trisporic acid can induce the formation of zygophores in the mucorales, but only a species-specific ratio of precursors (sex-specific pheromones) causes the directed growth of a zygophore toward its mate. This is the first plausible explanation of why so many different sex-specific compounds do the same thing (stimulate the development of zygophores) in the mucorales. Our speculation is consistent with recent studies in Oriental fruit moths which reveal that minor components among the pheromones emitted by females are involved in directing the flight of males to the female (Linn et al., 1987).

In summary, we have (1) discovered three more trisporic acids in cultures of *B. trispora*, two being new compounds; (2) demonstrated that the ratio of trisporic acids differs in membrane-separated and combined mating type cultures; (3) found that trisporic acid-E(3*R*) is not metabolized significantly by combined mating type cultures; (4) partially characterized by spectroscopy the constitution, configuration, and conformation of three trisporic acids; and (5) speculated that *B. trispora* and *P. blakesleeanus* utilize different pheromones to regulate an early event (possibly zygotropism) in sexual development.

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Registry No. Trisporic acid-E(3*R*), 90902-24-2; trisporic acid-E(3*S*), 119719-95-8; trisporic acid-D(2*S*), 119681-15-1; methyl trisporate-E(3*R*), 119923-87-4; methyl trisporate-E(3*S*), 120019-44-5; methyl trisporate-D(2*S*), 119923-88-5.

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[5] One hundred times more trisporic acid-D and 50 times more trisporic acid-E(3*R*) than trisporic acid-C are required for a minimal response in the *Mucor* bioassay for zygophore development (DeHaven, 1973). It was only after discovering that the methyl ester derivative of trisporic acid-E(3*R*) had (+) pheromone activity in zygophore bioassays with both *Mucor* and *Phycomyces* that Miller and Sutter (1984) determined its structure.

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Structure of a Second Crystal Form of Bence-Jones Protein Loc: Strikingly Different Domain Associations in Two Crystal Forms of a Single Protein†

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ABSTRACT: We have determined the structure of the immunoglobulin light-chain dimer Loc in a second crystal form that was grown from distilled water. The crystal structure was determined to 2.8-Å resolution; the *R* factor is 0.22. The two variable domains are related by local 2-fold axes and form an antigen binding "pocket". The variable domain–variable domain interaction observed in this crystal form differs from the one exhibited by the protein when crystallized from ammonium sulfate in which the two variable domains formed a protrusion (Chang et al., 1985). The structure attained in the distilled water crystals is similar to, but not identical with, the one observed for the Mcg light-chain dimer in crystals grown from ammonium sulfate. Thus, two strikingly different structures were attained by this multisubunit protein in crystals grown under two different, commonly used, crystallization techniques. The quaternary interactions exhibited by the protein in the two crystal forms are sufficiently different to suggest fundamentally different interpretations of the structural basis for the function of this protein. This observation may have general implications regarding the use of single crystallographic determinations for detailed identification of structural and functional relationships. On the other hand, proteins whose structures can be altered by manipulation of crystallization conditions may provide useful systems for study of fundamental structural chemistry.

Immunoglobulin light chains have an N-terminal variable (V) and a C-terminal constant (C) domain of about 100 residues each. These two domains are linked by a flexible "switch" peptide that makes movement of the domains possible. In the crystal structures of the Mcg (λ_V) light-chain dimer (Schiffer et al., 1973) and structurally analogous antigen binding fragments (Fab) [for a review, see Davies and Metzger (1983)], local 2-fold axes of symmetry, one relating the variable domains and one relating the constant domains, were identified. The angle between these dyads was called the "elbow" bend.

The Loc protein is a Bence-Jones protein consisting of two covalently linked λ_I immunoglobulin light chains (Zhu et al., 1983), which are disulfide bonded at their penultimate residues. In previous work, we determined the structure of the Bence-Jones protein Loc as crystallized from ammonium sulfate (Chang et al., 1985). In this structure, an unusual association of the V domains was found. Instead of the concave hapten binding cavity found in other light-chain dimers and Fabs, we observed a protrusion resulting from V domains that are related to each other by a 2-fold axis and a translation of 3.5 Å. Because of this unusual conformation of the antigen binding site, we again determined the structure of the same protein, but this time used a crystal form grown from distilled water. The Loc structure we are reporting here is more similar to the "usual" conformation with a local 2-fold and a hapten binding cavity between the V domains. Implications for structural studies of finding two structures for a molecule when it is crystallized from different solvents will also be discussed.

Materials and Methods

Preparation of Crystals. To prepare crystals, previous procedures (Chang et al., 1985) were slightly modified as follows: Crystals were grown at 4 °C by dialyzing 50–100-μL samples in dialysis buttons, at a concentration of 40 mg/mL protein [assuming A_{280}(1 mg/mL) = 1] in 0.3 M NaCl and 0.05 M Tris (pH 7.4) against distilled water; the pH was adjusted with NaOH or $Ca(OH)_2$ to 6.5. The crystals were then transferred into 16% poly(ethylene glycol) (PEG) 8000 in 0.05 M cacodylate, pH 6.5.

Heavy-Atom Derivatives. The exchange of the distilled water mother liquor for 16% PEG made the preparation of several heavy-atom derivatives possible; crystals in distilled water dissolved when salt solutions were added. Five heavy-atom derivatives were used to determine the phases to 5-Å resolution; these were $K_2Pt(CNS)_6$, [*p*-(hydroxymercuri)-phenyl]sulfuric acid, methylmercury chloride (MeHgCl), a covalent mercury derivative with the mercury inserted into the interchain disulfide between the two monomers (S–Hg–S), and the MeHgCl, S–Hg–S double derivative.

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