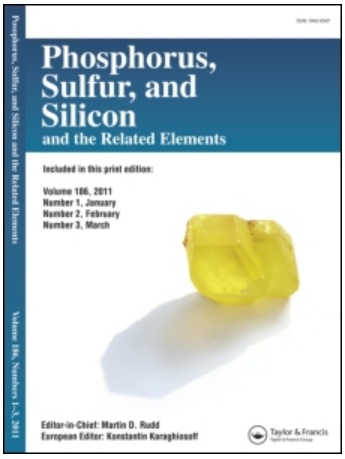

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Synthesis of Novel Heteroarotinoids with Receptor Activation Capabilities and Tgase Activity. Single Crystal Analysis of (*E*)-4-[(2,3-dihydro- 2,2,4,4,-tetramethyl-2*H*-1-benzo- [*b*]thiopyran-6-Yl)- 1-propenyl]-2-methylbenzoic Acid

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Synthesis of Novel Heteroarotinoids with Receptor Activation Capabilities and Tgase Activity. Single Crystal Analysis of (*E*)-4-[(2,3-dihydro-2,2,4,4,-tetramethyl-2*H*-1-benzo- [*b*]thiopyran-6-Yl)-1-propenyl]-2-methylbenzoic Acid

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*The syntheses of ethyl (E)-4-[(2,3-dihydro-2,2,4,4-tetramethyl-2H-1-benzo[b]-thiopyran-6-yl)-1-propenyl]-2-methylbenzoate (**1**) and (E)-4- [(2,3-dihydro-2,2,4,4-tetramethyl-2H-1-benzo[*b]*thiopyran-6-yl)-1-pro- penyl]-2-methylbenzoic acid (**2**) have been achieved. In comparison to ester **1**, acid **2** exhibited greater efficacy in activating RARα, RARβ, and RARγ as well as greater potency in activating RARα and RARβ. Interestingly, both the ester **1** and acid **2** exhibited nearly equal potency in activating RARγ. Both compounds also induced tissue transglutaminase (TGase)*

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*activity approximately 50% of the level induced by trans-retinoic acid. An X-ray diffraction analysis of (E)-**2** revealed the aryl rings as nearly orthogonal [74.0(2)°] and a torsional angle of 46.5(2)° between the thiochroman group and the propenyl group. Conjugation between such groups may not be a stringent requirement for receptor activation.*

Keywords Heteroarotinoids; NMR analyis; receptor activation; TGase activity; X-ray data

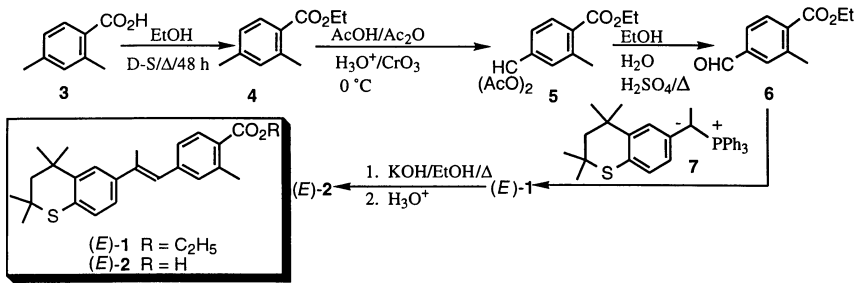
INTRODUCTION

Retinoids have been described as key agents in the differentiation of cells and could be important players in cancer chemotherapy.[1,2] Arotinoids are synthetic retinoids with at least one aromatic ring in the system,[2] and heteroarotinoids have a similar structural unit but also possess a heteroatom in a partially saturated ring usually fused to an aromatic ring.[3] We have shown that certain heteroarotinoids can activate retinoic acid receptors (RARs)[3f] and that selected members of this family of retinoids have anticancer properties.[3a–g] We report herein the synthesis of two novel heteroarotinoids, namely one with a terminal carboxyl group and the ester precursor thereof and possessing an adjacent methyl group. The rationale for these materials is based on biological data obtained from lead compounds.[3a–e] Moreover, an assessment of the methyl group on the aryl ring to alter receptor activation (since the group is near the CO_2Et terminus) was envisioned. Preliminary data indicate that receptor activation and ability to express TGase activity are related to the terminus (carboxyl versus ester) on the heteroarotinoid.[3f]

RESULTS AND DISCUSSION

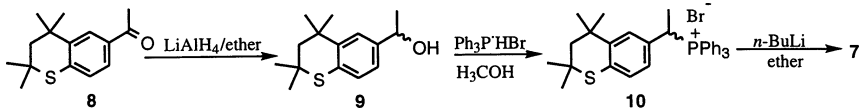
Chemistry

Syntheses of the targets **1** and **2** (Scheme 1) were initiated from **3** which was esterified under slightly different conditions than would be considered standard. A Dean-Stark trap was required to remove water and a long reaction time since the carboxyl group in **3** was somewhat hindered by the adjacent methyl group. Regiospecific oxidation of ester **4** was achieved with CrO_3 in the presence of Ac_2O which converted the acetal formed in situ to diacetate **5**. Acidification of **5** gave aldehyde **6** which was used immediately in the Wittig reaction with **7** to generate ester (*E*)-**1** (40%). Purification of (*E*)-**1** was realized only by chromatography and recrystallization to free the ester from several unknown impurities. Careful saponification of (*E*)-**1** produced acid (*E*)-**2** in



SCHEME 1

near quantitative yield. The Wittig reagent **7** was prepared as outlined (Scheme 2) starting from **8** which was reduced to the corresponding alcohol **9**. Phosphorylation of **9** occurred with triphenylphosphine hydrobromide in methanol to generate salt **10**. The generation of **7** had to be accomplished at −78°C in ether before the addition of **6**. The mixture of **7** in ether-hexane was stable only at −78°C, and **7** was used at once. Wittig reagent **7** was prepared (Scheme 2) starting from **8** which with **6** to obtain ester (*E*)-**1**.



SCHEME 2

Biology

In view of our previous findings that certain heteroarotinoids can be retinoid acid receptor (RAR) active and can alter TGase induction,[3f] it seemed prudent to assess in a preliminary manner such activities in (*E*)-**1** and (*E*)-**2** in an effort to ascertain the influence of such action by an ester group versus an acid group. The methodology and rationale for the receptor activation and TGase assays have been well documented.[3f] Since the potencies of the 9-cis-retinoic acid (9-*c*-RA) control assay vary from 1.6-fold to 0.3-fold, any difference in this range cannot be considered significant. However, the 7-fold and 27-fold differences in the RARγ and RARβ potencies for **1** and **2** can be considered significant. Activity data in Table I are based on the supposition that a minimum concentration of an agent for activation of a receptor reflects its best potency which is clearly in favor of acid (*E*)-**2**, especially for RARα and RARβ activation, even when compared to the standard 9-*c*-RA. Consequently, the carboxyl group may be much more important than an ester

TABLE I Potency of RAR Activation and TGase Inducement by (*E*)-1 and (*E*)-2

Agent	Efficacy[a]	Potency (nM)[b]	Potency (nM) of 9-*c*-RA	TGase[c]
(*E*)-**1**	33	RARα-890	RARα-200	0.52
	52	RARβ-300	RARβ-50	
	76	RARγ-420	RARγ-53	
(*E*)-**2**	47	RARα-33	RARα-320	0.56
	68	RARβ-43	RARβ-83	
	141	RARγ-640	RARγ-17	

[a]Percent maximal activity of the test compound/precent maximal activity of 9-*c*-RA (100).

[b]EC_{50} is the concentration required to produce 50% maximal activity. Values for 9-*c*-RA and **1** and **2** were calculated.

[c]Ratio of TGase activity (in nM) of heteroarotinoid to that of *t*-RA.

function in this type of receptor activation.[4] The TGase activity for both compounds was approximately 50% of that of *trans*-retinoic acid (*t*-RA), an indication of efficacy of the agents compared to the standard. The ability to induce TGase is a well-known marker of retinoid efficacy in leukemia cells.[3d,3e]

It has been recently recognized that RARγ activation requires a ligand which interacts in a special conformation.[3f] The 76% and 141% efficacy of RARγ activation by **1** and **2**, respectively, demonstrate that while both compounds interact with RARγ, acid **2** has a 2-fold higher efficacy. Since both of these efficacies were normalized to that of 9-*c*-RA, this 2-fold difference is significant. The potencies of concentrations 420 nM and 640 nM for **1** and **2**, respectively, however, cannot be considered significantly different, given the variation in the receptor potencies for the control 9-c-RA. Therefore, while **1** and **2** have similar potencies for RARγ, **2** is significantly more activating of RARγ as expected since the terminal carboxyl group is apparently required to form a "salt bridge" at the terminus of the ligand.[3f] Consequently, it is conceivable that the lower activation by ester **1** is due to the slow conversion by esterases to the carboxyl group in the cells. We have previously shown that a flexible form of a ligand for RARγ activation is likely a more accurate representation of the ligand bound to RARγ than is a rigid form.[3f] Thus, the degree of bond rotational freedom of groups attached to the double bond in **1** or **2** is presumably sufficient to promote receptor activation. Ester **1** and acid **2** induce RARγ with similar potencies and induce TGase to similar degrees, which is consistent with the prior observations that RARγ mediates the effects of retinoic acid on TGase expression.[8] This study is the first where comparisons have been made regarding activation of RARs by hetero-arotinoids. The X-ray data of (*E*)-**2** indicates

that in the solid state much conjugation between the aryl rings is not prevalent, but such a requirement in solution for RAR activation is unknown.

X-Ray Crystallographic Analysis of the Target Ligand (*E*)-2

Since the absolute structures of heteroarotinoids have rarely been determined, an X-ray diffraction analysis of (*E*)-**2** was performed (Table II). Crystals were of the triclinic space group, and the structure consisted of dimers which uniquely employed hydrogen bonding through the carboxyl function. Intramolecular and intermolecular O—H bond distances of 0.905(4) Å and 1.789(4) Å, respectively, were found and are typical of such hydrogen bonding. The bond angles of O—H···O, C—O—H, and C=O···H were 155.6(4)°, 109.8(4)°, and 122.7(4)°, respectively, and are also reasonable for such H-bonding. No long-range bondings were noted for other groups in (*E*)-**2**. Interestingly, the C6-C14-C15 bond angle [115.6(5)°] was compressed by the large cis-arranged aryl ring as revealed by the larger C14-C16-C17 bond angle [130.8(5)°] created presumably by ortho hydrogens at C22 (or C18) experiencing repulsion from the methyl hydrogens of the propenyl group.

Three torsional angles considered critical for the structure were measured. The angle defined by the thiochroman ring system and the phenyl ring at the end of the linker group was nearly orthogonal at 74.0(2)° (Figure 1). This angle is reminiscent of that found for another heteroarotinoid[5] but differs from that found for *trans*-stilbene.[6] A more exact torsional angle between the phenyl rings in *trans*-stilbene was reported later to be 5.2°.[7] The angle between the

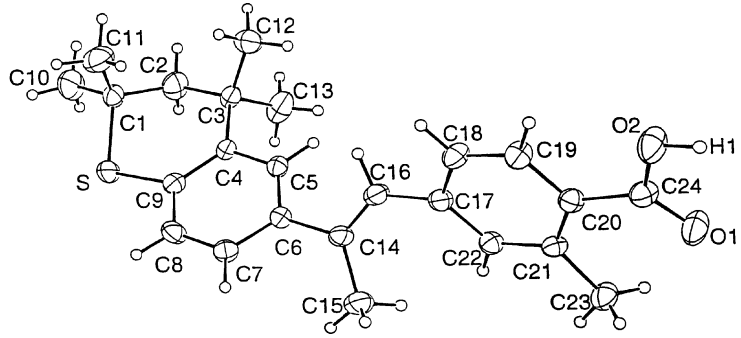


FIGURE 1 ORTEP drawing of (E)-2

TABLE II Crystal and Refinement Data for (*E*)-2

Formula	$C_{24}H_{28}O_2S$
fw	380.54
Crystal size, mm	0.18 × 0.30 × 0.48
Crystal color	Colorless
Crystal mount	On glass fiber encapsulated in epoxy
a, Å	6.7122(18)
b, Å	7.9440(20)
c, Å	21.173(50)
α, deg	90.670(20)
β, deg	90.040(20)
γ, deg	21.173(5)
Cell detn, refls	25
Cell detn, 2θ range, deg	20–22
d (calcd), g cm^{-3}	1.208
Space group	P-1
Z	2
F (000)	408.45
Radiation	MoKα, graphite monochromated
γ, Å	0.71073
Temp, K	293
Linear abs coeff, mm^{-1}	16.2
Diffractometer	Enraf-Nonius CAD-4
Scan technique	θ-20
Scan speed, deg min^{-1}	4–16 (in omega)
Scan width, deg	1.0 + 0.35 tan θ
2θ range, deg	4–50
h, k, l ranges	−7,7; 0,9; −25, 25
Exposure time, hrs	25.7
Std refl indices	0,−3,7; 3,−3,−2; 0,−3,−6
Drift of stds, %	1.0
Absorbtion correction	Gaussian by faces
Absorbtion, range	0.96–0.97
Refl meas	3957
Unique refls	3665
R for merge	0.013
Data with I > 2.5 δ (I)	1641
Solution method	Direct methods
Parameters refined	244
R(F^2), Rw(F^2)	0.057, 0.078
GOF	1.18
P, w-1 = [δ^2(I) + pI^2]/4F^2	0.05
Largest Δ/δ	0.000
Extinction correction	None
Final diff man, e $Å^{-3}$	−0.23(5), 0.24(5)
Programs	NRC386 (PC version of NRVAX)*
Scattering factors	Internat. Tables for Crystallography, Vol. 4
H atom treatment	Methyl positions from maps, subsequently; idealized so that C—H = 0.95 Å & U = Uc + 0.01
O atom treatment	Hydroxyl pos. from maps, subsequently; thermal Parameter U = U° + 0.01

*NRCVAX—An Interactive Program System for Structure Analysis, E. J. Gabe, Y. LePage, J. P. Charland, F. L. Lee, and P. S. White, *J. Appl. Cryst.*, **22,** 383 (1989).

aryl ring of the thiochroman unit and the propenyl linker was 46.5(2)° in (*E*)-**2**. In contrast, an angle of 28.9(3)° was present between the propenyl linker and the single phenyl ring attached to the linker in (*E*)-**2**. Thus, in the solid state there is little indication that conjugation exists between the two aryl rings and the linker moiety. These data may provide additional insight regarding future studies of activation of RARγ by synthetic ligands in terms of orientation of groups within the ligand.

In summary, the syntheses of two new heteroarotinoids are herein reported. It was also been found that a difference in receptor activation capability prevails in certain heteroarotinoids with slightly differing terminal groups, that is an ester versus an acid group. This is presumed to be due to the requirement for formation of a salt bridge [CO_2^-] for RARγ activation by a ligand.[3f] In addition, the arrangement between the two aryl rings in the title acid suggests that strong conjugation between the rings in such systems, via the linker group, may not be absolutely critical for receptor activation as well as TGase activity.

EXPERIMENTAL

General

Melting points were taken on a Perkin-Elmer 681 unit and were uncorrected. All NMR spectra were recorded on a Varian 300 MHz or Varian 400 MHz spectrometer and registered as δ or ppm, respectively, from TMS. Both ^{1}H and ^{13}C spectra were taken at 299.4/399.95 MHz and 75.43/100.6 MHz, respectively. IR data were taken on a Perkin-Elmer 2000 FT-IR unit. Elemental analyses were obtained by Atlantic Inc., Norcross, Georgia. All chromatographic separations were effected with silica gel (Baker, 40 m, 60Å, flash). Storage of all intermediates and final products in the cold/dark minimized decomposition.

*Ethyl (*E*)-4-[(2,3-Dihydro-2,2,4,4-tetramethyl-2*H*-1-benzothiopyran-6-yl)-1-propenyl]-2-methylbenzoate (1)*

A solution of *n*-butyllithium in hexane (0.15 mL, 1.56 mmol, 10 *M*) was added dropwise (5 min, N_2) to a suspension of the salt **10** (0.82 g, 1.43 mmol) in dry ether (25 mL). After stirring for 1 h at RT, the mixture containing **7** was cooled (−78°C, dry ice-acetone, 10 min), and a solution of ethyl 2-methyl-4-formylbenzoate (**6**, 0.25 g, 1.3 mmol) was added dropwise (0.25 h). The mixture was stirred (0.75 h), and then it was allowed to slowly warm to RT (1 h). The pale yellow mixture was stirred

(48 h), and then the mixture was filtered to remove the triphenylphosphine oxide, the latter residue being washed with dry ether (100 mL). This washing and the filtrate were combined, dried (Na_2SO_4), and evaporated to an oil which was chromatographed (hexane:ether, 98:2). Fractions containing crude **1** were combined and evaporated to an oil which was treated with boiling ethanol (95%). Chilling (dry ice bath) the final solution for 24 h induced precipitation. Filtration and recrystallization (95% ethanol) of the solid gave ester (*E*)-**1** (0.21 g, 40%), mp 77–79°C. IR (KBr) 1705 cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.40 [m, 3 H], 1.44 [s, 12 H], 1.98 [s, 2 H], 2.28 [s, 3 H], 2.63 [s, 3 H], 4.36 [m, 2 H], 6.77 [m, 1 H], 7.11 [m, 1 H], 7.25 [m, 3 H], 7.53 [s, H], 7.93 [m, 1 H]; ^{13}C NMR ($DCCl_3$) ppm 14.3, 17.6, 21.9, 31.6, 32.6, 35.6, 42.1, 54.4, 60.6, 123.7, 124.4, 125.9, 126.3, 127.9, 130.5, 130.6, 132.4, 139.1, 140.0, 140.2, 142.0, 142.4, 167.5. MS (EI) $C_{26}H_{32}O_2S$ (M^+): 408.2123. Found: 408.2114. $C_{26}H_{32}O_2S$ requires: C, 76.43; H, 7.89; found: C, 76.32; H, 7.96.

(E)-4-[(2,3-Dihydro-2,2,4,4-tetramethyl-2H-1-benzothiopyran-6-yl)-1-propenyl]-2-methylbenzoic Acid (2)

A solution of (*E*)-**1** (0.32 g, 0.80 mmol) in water (5 mL) and ethanol (20 mL, 95%) containing KOH (0.45 g, 8.03 mmol) was boiled (2 h), then stirred at RT (12 h), and finally chilled to 0°C. Dropwise addition of HCl (~20 mL, 2 *N*) resulted in the precipitation of slightly crude (*E*)-**2** as a white solid (0.30 g, 99%). Recrystallization (95% ethanol) of the solid gave (*E*)-**2** as colorless crystals (~qt), mp 178–180°C. IR (KBr) 3450 (HOC=O), 1690 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.39 [s, 12 H], 1.95 [s, 2 H], 2.24 [s, 3 H], 2.55 [s, 3 H], 6.87 [m, 1 H], 7.06 [m, 1 H], 7.31 [m, 3 H], 7.64 [m, 1 H], 7.86 [d, 1 H]; ^{13}C NMR (DMSO-d_6) ppm 17.1, 21.3, 31.1, 32.3, 35.1, 41.8, 53.6, 123.6, 124.3, 125.4, 126.2, 127.3, 127.8, 130.3, 138.1, 139.1, 139.5, 141.2, 142.0, 168.2. MS (EI) $C_{24}H_{28}O_2S$ (M^+): 380.1812. Found: 380.1810. $C_{24}H_{28}O_2S$ requires: C, 75.75; H, 7.42; found: C, 75.47; H, 7.49.

Ethyl 3,4-Dimethylbenzoate (4)

A solution of 3,4-dimethylbenzoic acid (**3**, 6.0 g, 39.9 mmol), absolute ethanol (80 mL), and benzene (150 mL) with H_2SO_4 (1.5 mL) as a catalyst were heated in a Dean-Stark trap (48 h). After cooling, the solution was diluted (water, 100 mL) and extracted (ether, 3 × 40 mL). The extracts were combined and washed with saturated $NaHCO_3$ (3 × 40 mL), water (2 × 50 mL), and brine. Evaporation of the solvent and distillation (65°C/0.25 mm) of the oil gave ester **4** (6.3 g, 88%) which was used immediately to prepare **5**. Spectral data for **4**: IR 1710 cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.39 [t, 3 H], 2.31 [s, 3 H], 2.58 [s, 3 H], 4.30 [m,

2 H], 7.05 [m, 2 H], 7.85 [m, 1 H]; ^{13}C NMR ($DCCl_3$) ppm 14.3, 21.3, 21.7, 60.4, 126.3, 126.9, 130.6, 132.4, 140.1, 142.2, 167.5.

Ethyl 2-Methyl-4-formylbenzoate (6)

A solution of ester **4** (1.6 g, 8.9 mmol), glacial acetic acid (20 mL), and freshly distilled acetic anhydride (20 mL) with H_2SO_4 (1.5 mL) was stirred at RT (0.25 h) after which time the solution was cooled to 0°C. The temperature was maintained <5°C (1 h) as CrO_3 (1.6 g, 16.8 mmol) was added over 0.5 h. The resulting mixture was stirred at RT for 8 h after which the mixture was treated very *cautiously* with ice water (150 mL) and then ether (40 mL). Extracts ($HCCl_3$, 3 × 50 mL and ether, 1 × 50 mL) were combined with the original organic layer and washed with saturated $NaHCO_3$ (3 × 40 mL), water (1 × 50 mL), and brine. After drying (Na_2SO_4), the solvent was evaporated to yield the diacetate **5** as an orange oil (1.9 g, 76.8%). The oil **5** was air sensitive and thus was dissolved immediately in ethanol (15 mL) to which was added dropwise (10 min; slightly exothermic) water (10 mL) and conc H_2SO_4 (0.7 mL). Additional water (150 mL) was added to the solution at RT, and two phases separated. Extracts ($HCCl_3$, 5 × 40 mL) were combined with the original organic layer and washed with saturated $NaHCO_3$ (4 × 35 mL), water (50 mL), and brine. The dried (Na_2SO_4) solution was evaporated to a light brown oil which was chromatographed (hexanes:ether, 8.5:1.5) to yield **6** as a pale yellow oil (0.36 g, 21%) which solidified at 0°C and remelted near RT.[9] IR (neat) 2720 (H—CO), 1725 (EtOC=O), 1710 (HC=O) cm^{-1}; ^{13}C NMR ($DCCl_3$) ppm 14.1, 21.3, 61.2, 126.5, 130.8, 132.4, 135.2, 137.9, 140.5, 166.7, 191.7. The ^{1}H NMR spectrum of **6** has been reported.[7] Since **6** oxidized rapidly, it was used at once to make (*E*)-**1**.

2,2,4,4-Tetramethythiochroman-6-(1-hydroxyethane) (9)

A solution of ketone **8**[3c] (5.8 g, 23.3 mmol) in anhydrous ether (25 mL) was added (0.25 h, N_2) to a stirred suspension of $LiAlH_4$ (1.42 g, 37.36 mmol) in dry ether (15 mL). After stirring the grey suspension at reflux (24 h), the mixture was cooled to RT, and EtOAc (10 mL) was added very slowly with cooling (<5°C). A solution of 5% HCl (50 mL) was added slowly with stirring. Ether (50 mL) was then added, and, after extraction (ether, 4 × mL), the extracts and original organic layer were combined and washed with saturated $NaHCO_3$ (3 × 40 mL), water (50 mL), and brine. Drying (Na_2SO_4) and distillation gave **9** as a thick, yellow oil (50–55°C/0.3 mm; 5.6 g, 96%) which was used at once without further purification to prepare **10**. Data for **9** were: IR (neat) 3350 (O—H) cm^{-1}; ^{1}H NMR ($DCCl_3$) δ 1.41 [s, 6 H], 1.43 [s, 6 H], 1.53 [m, 3 H], 1.96 [s, 2 H], 4.84 [m, 1 H], 7.15 [m, 2 H], 7.42 [m, 1 H]; ^{13}C

NMR ($DCCl_3$) ppm 24.9, 31.6, 32.5, 35.6, 41.9, 54.4, 70.3, 123.1, 123.9, 124.9, 128.01, 128.08, 142.7.

1-[(2,2,4,4-Tetramethylchroman-6-yl)ethyl]triphenylphosphonium Bromide (10)

A solution of alcohol **9** (5.5 g, 21.96 mmol) and triphenylphosphine hydrobromide (8.9 g, 26.4 mmol) in methanol (125 mL) was stirred at RT (24 h, N_2). Evaporation of the pale yellow solution and repeated trituration of the clear oil obtained with dry ether induced solidification. Stirring the suspension of the light yellow solid in dry ether (100 mL) for 4 h gave a very light yellow solid which was filtered off and dried (110°C/2 mm); yield 11.1 g (88%), mp 140–150°C. The salt **10** was used immediately to prepare anion **7** which in turn was used to prepare **1**. Data for **10** were: ^{1}H NMR ($DCCl_3$) δ 1.12 [s, 3 H], 1.23 [s, 3 H], 1.37 [s, 6 H], 1.38–1.85 [m, 2 H], 1.87 [m, 3 H], 6.48–6.54 [m, 1 H], 6.60 [m, 2 H], 6.89 [m, 1 H], 7.77 [m, 15 H]; ^{13}C NMR ($DCCl_3$) ppm 19.6, 31.4, 31.9, 32.5, 32.7, 35.5, 54.1, 70.3, 117.2, 118.3, 126.9, 128.0, 129.8, 130.0, 130.1, 130.2, 130.4, 130.5, 132.0, 133.2, 133.3, 134.5, 134.6, 134.7, 135.1, 135.2.

*Crystallographic Experimental Data for (*E*)-2*

Suitable crystals of the acid (*E*)-**2** were obtained by slow diffusion of pentane into a saturated solution of the acid in absolute ethanol, followed by slow evaporation of the ethanolic solvent. The crystals were triclinic and of the space group P1. Data collection was accomplished with an ENRAF-Nonius CAD-4 diffractometer with monochromated Mo Kα ($\lambda = 0.71073$ Å). Crystals were mounted on a glass fiber with silicone and then coated with epoxy to prevent decomposition. The cell dimensions were determined by a least squares fit to 25 reflections with $\pm 2\theta$ in the range of 20–22°. The structure was solved by direct methods using the program NRC386.[10]

The structure was refined by a full-matrix least squares method which minimized $\Sigma\omega(\Delta F)^2$. A total of 3957 reflections were taken. The hydrogen belonging to the carboxyl group was located on a difference map and then constrained to that position. Methyl hydrogens were also located by a difference map and then normalized to idealized positions with C—H = 0.95 Å. All other hydrogen atoms were generated and constrained to idealized positions with C—H = 0.95 Å. All hydrogen atoms were assigned isotropic U values of 0.01 greater than that of the attached atom. The structure refined to a final R factor of 0.057.[a]

[a]Tables of all X-ray data have been deposited with the Cambridge Crystallographic Data Centre (CCDC), United Kingdom. CCDC 213322.

Biological Assays

The technique for assaying for transglutaminase (TGase) activity has been completely described.[3d] The methodology for determining retinoic acid receptor activation has also been well delineated.[3e]

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