Notes

Retinoylserine and Retinoylalanine, Natural Products of the Moth *Trichoplusia ni*

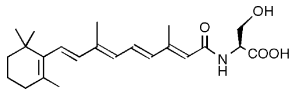
Barbara Rogge,[†,‡,§] Yasuhiro Itagaki,[⊥] Nathan Fishkin,[⊥] Ester Levi,[†] Ralph Rühl,[∥] San-San Yi,[▽] Koji Nakanishi,[⊥] and Ulrich Hammerling*[,†]

Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, Department of Chemistry, Columbia University, New York, New York 10027, Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary, Institute of Nutritional Science, University of Potsdam, Potsdam, Germany, and Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

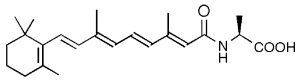
Received September 30, 2004

Insect cells convert vitamin A into a number of retinoids that are evolutionarily conserved with those of mammalian cells. However, insect cells also produce additional natural retinoids. Namely, two retinoic acid peptides, *N-trans*-retinoylserine (**1**) and *N-trans*-retinoylalanine (**2**), have been isolated from a cell line of the common cabbage looper, *Trichoplusia ni*. These are the first examples of naturally occurring retinoic acid linked to amino acids through an amide bond; the amino acid moieties are depicted in the more common L-configuration, although the absolute configuration was not determined due to the minuscule sample amount.

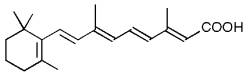
In this report, we describe the characterization and synthesis of *N-trans*-retinoylserine (**1**) and *N-trans*-retinoylalanine (**2**) isolated from insect cells (Figure 1). Vitamin A is used in higher animals as a precursor for retinaldehyde, the universal visual pigment, and for retinoic acid (**3**), which serves as an important transcriptional activator in development and homeostasis. Furthermore, hydroxylated forms of vitamin A, and indeed vitamin A itself, play a broad role in signal transduction. Insect cells convert vitamin A into a similar, evolutionarily conserved spectrum of retinoids. However, the usage of vitamin A or all-*trans*-retinol (**4**) in insects is an underexplored area. With the exception of retinal, which represents the universal pigment of all eyes[1] for both invertebrates and vertebrates, the importance of retinol for insects is not known. Yet indirect signs of systemic vitamin A usage have been noted. These include observations that retinoids have profound effects on the development of invertebrates and vertebrates alike.[2] Since the RAR (retinoic acid receptor) and RXR (retinoid X receptor) classes of retinoid receptors[3,4] do not make an appearance in evolution until the vertebrate boundary, mechanisms other than retinoic acid-mediated gene regulation are implicated for invertebrates.[5] In fact, biochemical evidence in flies and moths shows that vitamin A is consistently metabolized to hydroxylated forms: 14-hydroxy-*retro*-retinol (**5**) (14-HRR, enantiomeric mixture) and 13,14-dihydroxyretinol (**6**) (DHR, configuration unknown).[6,7] Additionally, the rare metabolite anhydroretinol (**7**) (AR), first encountered in mammals,[8] was found in *Drosophila* cell lines, while the enzyme retinol dehydratase, used in AR biogenesis, was isolated and characterized from *Spodoptera frugiperda* cells.[9] Finally, retinol-binding sites on the conserved zinc-finger fold of the *Drosophila* kinases, cRaf (serine/threonine Raf kinase family member C) and PKC (protein kinase C), have been identified.[10,11] Although perhaps no more than fossil footprints, they hint at a primordial function of vitamin A in signal transduction. These observations suggest a picture of widespread vitamin A metabolism and usage in insects.



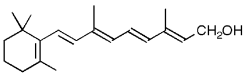
1. N-*trans*-retinoylserine



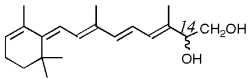
2. N-*trans*-retinoylalanine



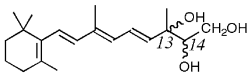
3. all-*trans* retinoic acid (RA)



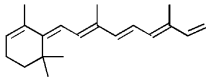
4. all-*trans* retinol



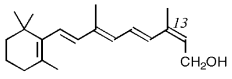
5. 14-hydroxy-*retro*-retinol (14-HRR)



6. 13,14-dihydroxyretinol (DHR)



7. anhydroretinol (AR)



8. 13-*cis*-retinol

While surveying the natural vitamin derivatives of a cell line derived from the moth *Trichoplusia ni* (Walker, 1858), we encountered retinoyl amides of the amino acids serine and alanine. Although retinoyl esters of a number of mammalian proteins were reported,[12,13] amino acid derivatives of retinoic acid as natural products have not been

* To whom correspondence should be addressed. Tel: (212) 639-7523. Fax: (212) 794-4019. E-mail: u-hammerling@ski.mskcc.org.
† Immunology Program, Memorial Sloan-Kettering Cancer Center.
‡ University of Potsdam.
§ Present address: Institute of Pathobiochemistry, University of Mainz, Mainz, Germany.
⊥ Columbia University.
∥ University of Debrecen.
▽ Molecular Biology Program, Memorial Sloan-Kettering Cancer Center.

10.1021/np0496791 CCC: $30.25
Published on Web 09/17/2005

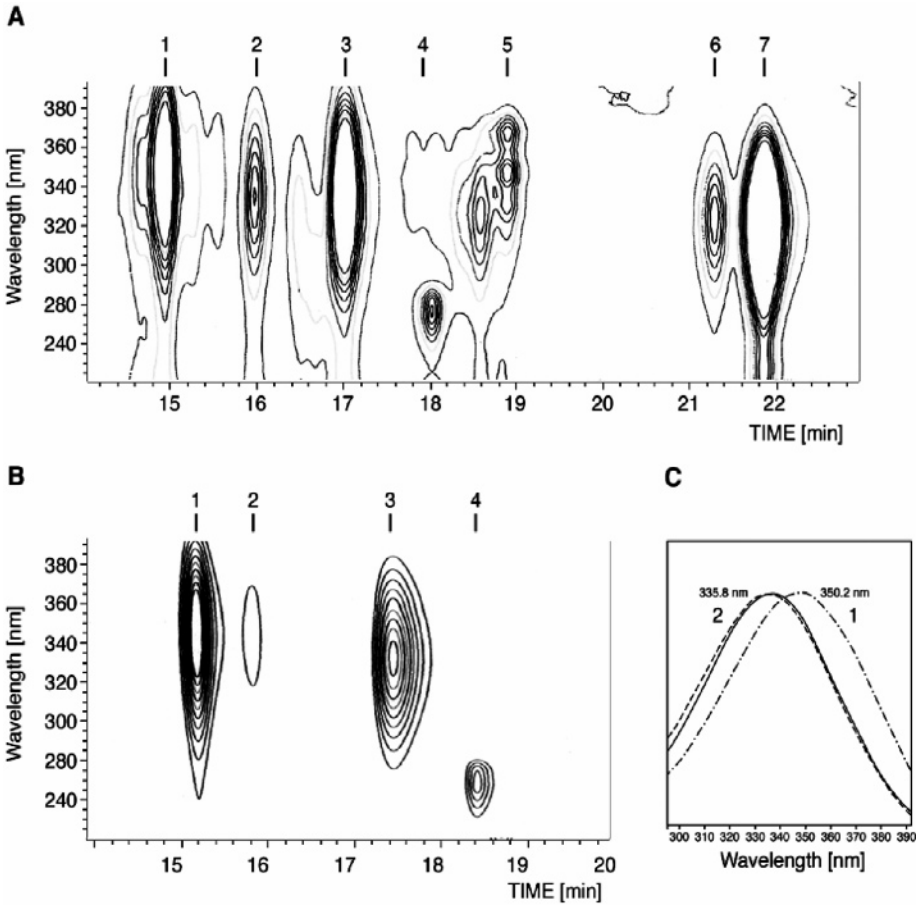


Figure 1. Contour plot chromatogram, C_{18} HPLC, of *Trichoplusia ni* Hi-5 retinoids. Cells were grown for 24 h in the presence of (A) retinoic acid (**3**) or (B) all-*trans*-retinol (**4**); inset C shows UV spectra in MeOH. HPLC peaks denote the following: peak 1, *N*-*trans*-retinoylserine (**1**), λ_{max} 350 nm (broken line in inset C); peak 2, *N*-*trans*-retinoylalanine (**2**), λ_{max} 336 nm (dashed line in inset C); peak 3, all-*trans*-retinoic acid (**3**), λ_{max} 336 nm (solid line in inset C); peak 4, butylated hydroxytoluene; peak 5, 14-HRR (**5**); peak 6, 13-*cis*-retinol (**8**); peak 7, all-*trans*-retinol (**4**). The shifts in UV_{max} of **1**, **2**, and **3** cannot be fully rationalized at this stage. The OH group in **1** could be contributing to the 14 nm red shift relative to **2** and **3**. It is possible that H-bonding partially reduces the bond order of the amide carbonyl, thereby increasing polyene conjugation with the amide nitrogen.

identified previously. Synthetic *N*-retinoyl amino acids were previously tested, though, for efficacy in controlling differentiation of epithelial cell lines.[14,15]

Cultured cells are normally supplied with vitamin A by fetal bovine serum. To create defined culture conditions, the Hi-5 insect cells were grown in the presence of 10 μM retinol. The retinoids produced by metabolic conversion of vitamin A were extracted with BuOH and separated by HPLC on a C_{18} column, using a H_2O/MeOH/$CHCl_3$ gradient. A contour plot chromatogram of Hi-5 retinol metabolites revealed, in addition to peaks 1 and 2, the following known retinoids: peak 3 (RA, all-*trans*-retinoic acid, **3**), peak 5 (14-HRR, **5**), peak 6 (13-*cis*-retinol, **8**), and peak 7 (*trans*-retinol, **4**) (Figure 1A). These were identified by their UV absorption spectra and comparison of HPLC retention times with reference standards.[6] DHR (**6**) was also observed in some instances.[7] The peak eluting at 18 min represents the antioxidant butylated-hydroxytoluene (BHT).

Peaks 1 and 2 represented two unknown compounds (**1** and **2**) with UV absorption maxima at 350 and 336 nm (Figure 1C) and retention times of 14.9 and 16.0 min, respectively. While retention times differed between HPLC runs, the retention time relative to the reference RA (**3**) was reproducible. Compound **1** preceded RA (**3**) (peak 3) by 2.2 min. Whether incubated with retinol for 3 or 24 h, the composition did not change qualitatively, but the amount of the hitherto unknown retinoids, notably **1** and **2**, increased prominently over 24 h. The fact that RA (**3**) also increased in quantity with extended incubation at the expense of retinol suggested the genesis of a derivative of RA (**3**). This was confirmed by substituting RA (**3**) for retinol in the cell culture medium, upon which no compound other than RA (**3**) and the two unknown retinoids **1** and **2** emerged in the extract (Figure 1B). Incubation of control cell lines with retinol, including a large number of mammalian lymphoblast and fibroblast cells lines as well as *Drosophila* S2M3 cells, yielded neither compound **1** nor **2**,[8] indicating that the two do not represent chemical artifacts but are specific for Hi-5 cells and are produced by biochemical conversions. Although insects are known to metabolize vitamin A, it was not known whether the bioconversion products include compounds **1** and **2**.

Upon scaling up the incubation and purifying the retinoic acid derivatives to homogeneity, protonated molecular ions were obtained at *m/z* 388 for compound **1** and *m/z* 372 for compound **2** by ESIMS. These protonated molecular ions were observed whether the incubations were carried out with retinol (**4**) or RA (**3**) as the substrate. The molecular ion *m/z* 387.2410 observed by HRFABMS corresponded to a molecular formula of $C_{23}H_{33}O_4N$ (calcd 387.2409). The UV of compound **1** (Figure 1C) suggested that the retinoid contained the full polyene chain of RA (**3**) and that the remaining mass was likely to be due to the presence of the amino acid serine. As the compound was resistant to alkali

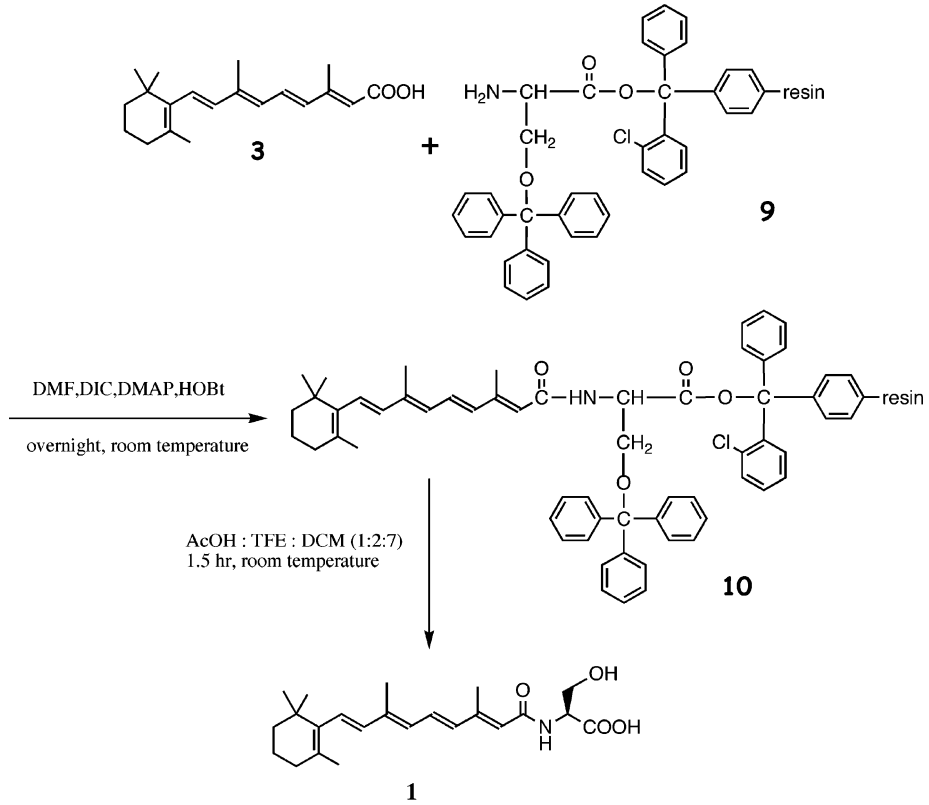


Figure 2. Synthesis of *N-trans*-retinoylserine (**1**) by condensation of retinoic acid (**3**) with resin-bound trityl L-serine.

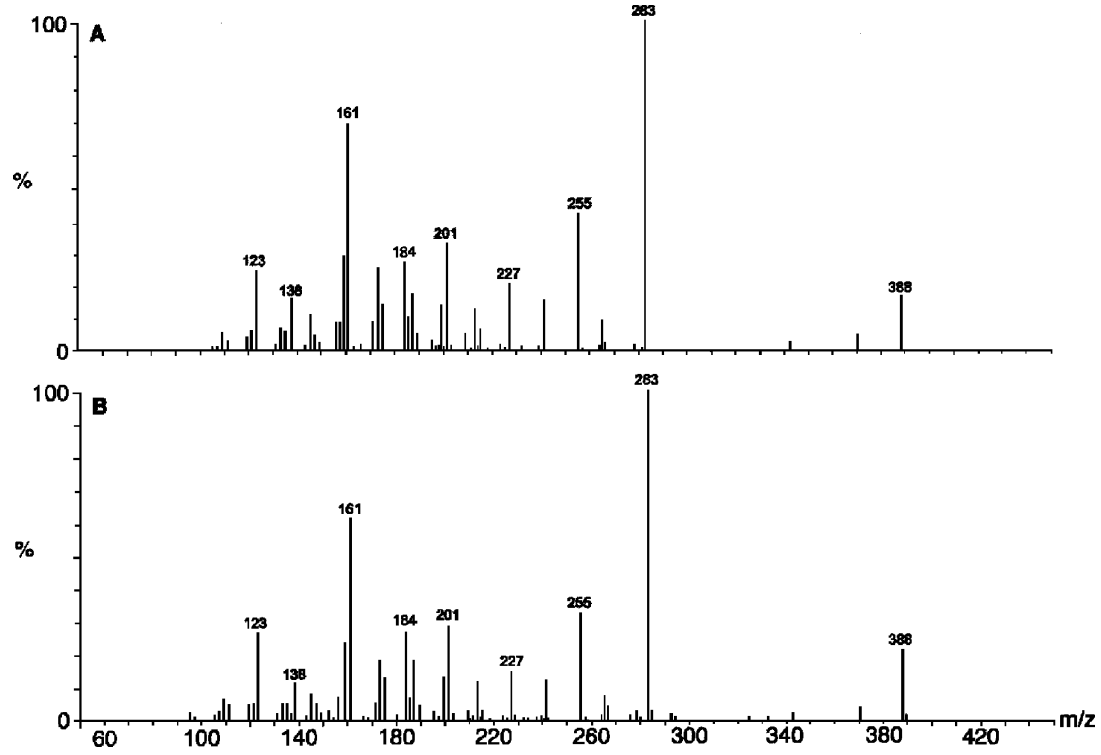


Figure 3. Protonated molecular ion ESIMS/MS spectra of natural (A) and synthetic (B) *N-trans*-retinoylserine (**1**).

treatment, attachment of serine to RA (**3**) was presumed to be through an amide, as opposed to an anhydride or ester bond.

Thus, *N-trans*-retinoylserine (**1**) was synthesized by condensation of RA (**3**) with trityl-protected, resin-bound 2-chlorotritylserine (**9**), as shown in Figure 2. The ESIMS/MS spectrum of both the natural product and synthetic *N*-retinoylserine showed the same fragmentation pattern (Figure 3).

The HRFABMS elemental composition of compound **2** was determined as $C_{23}H_{33}O_3N$ (obsd 371.2464, calcd 371.2461), corresponding to the molecular ion of *N-trans*-retinoylalanine, which was then synthesized by condensation of RA (**3**) with L-alanine methyl ester, followed by ester hydrolysis (Figure 4).

The identical ESIMS/MS spectra of natural and synthetic products established the structure of compound **2** as *N*-retinoylalanine (Figure 5). The absolute configuration

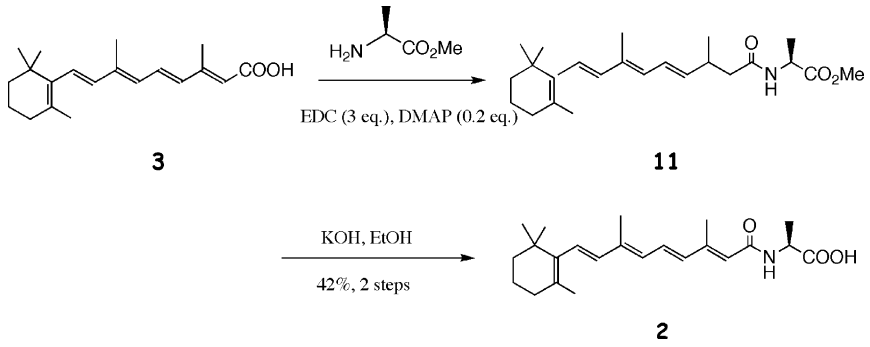


Figure 4. Synthesis of *N-trans*-retinoylalanine (**2**) by condensation of retinoic acid (**3**) with L-alanine methyl ester.

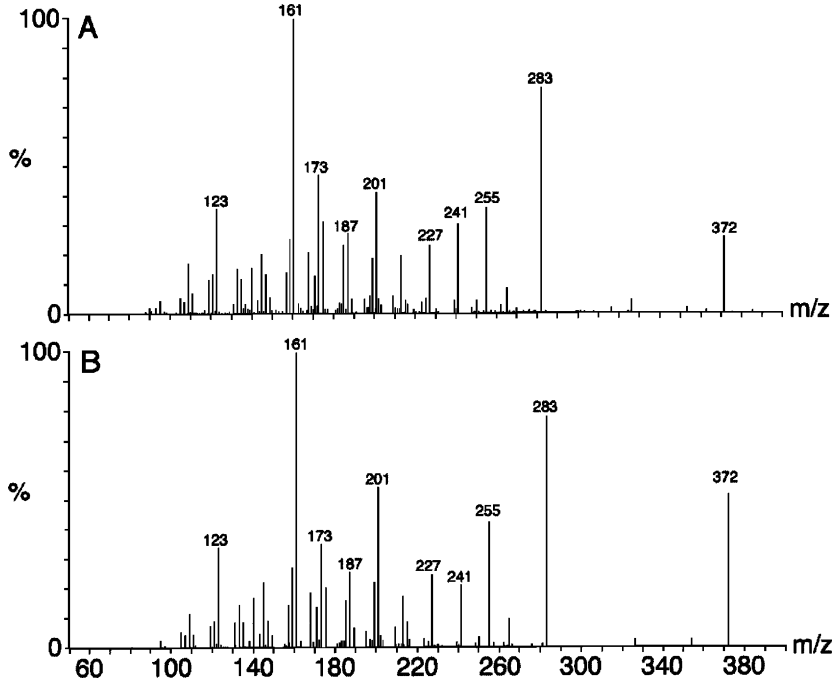


Figure 5. Protonated molecular ion ESIMS/MS spectra of natural (A) and synthetic (B) *N-trans*-retinoylalanine (**2**).

of the amino acid moieties could not be determined due to the miniscule amounts of the natural products isolated, but they are depicted with the more common L-configurations.

While the biological roles of *N-trans*-retinoylserine (**1**) and *N-trans*-retinoylalanine (**2**) are not known, as is indeed the case for most other retinoids found in insect cells, these may represent the prototypes of retinoyl peptides. In any event, the occurrence of these vitamin A derivatives, along with retinaldehyde, RA (**3**), 14-HRR (**5**), DHR (**6**), and AR (**7**),[6–9] bears witness to the richness of retinoid metabolism in invertebrates. The biochemistry of these metabolites represents a largely unexplored area of research that will yield important information regarding the unconventional, non-nuclear functions of vitamin A in the mammalian species[16] since invertebrates are likely to have vitamin A pathways that are conserved in higher organisms.

Experimental Section

General Experimental Procedures. HPLC was performed using an ODS column (Vydac, Hesperia CA, C_{18} 0.46 mm × 250 mm) and Waters Model 600E HPLC equipped with a Model 996 UV photodiode array detector. LR- and HR-FABMS were measured on a JEOL HX110A/110A tandem mass spectrometer using *m*-nitrobenzyl alcohol (NBA) as the matrix. ESI and ESIMS/MS were measured on a Micromass Q-TOF equipped with a Waters 2690 HPLC. Needle voltage was 3000 V, and the collision energy for MS/MS was 30 V. The collision gas was He. The ^{1}H NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer.

Isolation of *N-trans*-Retinoylamino Acids. The *Trichoplusia ni* cell line Hi-5 (Granados, R.R. US 5 300 435, 1994) was purchased from Invitrogen (Carlsbad, CA) and grown as suspension culture in serum-free Express Five medium at 27 °C to a density of 2 million cells per mL. All-*trans*-retinol (**4**), or alternatively RA (**3**), was added at a final concentration of 10 μM, and culturing was continued from 3 to 24 h. Control cultures of Hi-5 cells without added retinol yielded no discernible retinol metabolites. Cells were harvested, washed repeatedly with phosphate buffered saline, and extracted with n-BuOH in the presence of added BHT by the method of McClean et al.[17] Retinoids were resolved on a C_{18} column by HPLC using a H_2O/MeOH/$CHCl_3$ gradient as described.[6] A photodiode array UV detector was used to identify eluted retinoids. The C_{18} column was calibrated with authentic retinoids RA (**3**), all-*trans*-retinol (**4**), 14-HRR (**5**), and DHR (**6**) as standards. The molecular formulas of compound **1** and **2** were determined to be $C_{23}H_{33}0_4N$ (387.2419, calcd 387.2409) and $C_{23}H_{33}0_3N$ (371.2464, calcd 371.2461), respectively, by HRFABMS.

Synthesis of *N-trans*-Retinoylserine (1) (Figure 2). *N-trans*-Retinoylserine (**1**) was synthesized by the scheme shown in Figure 2: 0.25 mmol of L-Ser(Trt)-2-Cl-Trt resin (**9**) (AnaSpec, San Jose, CA) was swollen in 10 mL of CH_2Cl_2 for 10 min, drained, and reacted with 1 mmol of RA (**3**) dissolved in 4 mL of DMF in the presence of 1 mL of DIC (*N*,*N*-diisopropylcarbodiimide, Acros, Pittsburgh, PA), 20 mg of

DMAP ((dimethylamino)pyridine), and 135 mg of *N*-hydroxybenzotriazole hydrate (HOBt) (Novabiochem, San Diego, CA). A coupling efficiency close to 100% was achieved after 18 h. The resin was washed with DMF, and the reaction product (**10**) was cleaved from the resin by 1:2:7 mixtures of HOAc, 2,2,2-trifluoroethanol, and CH_2Cl_2 for 90 min at room temperature. Product **1** (161 mg of crude peptide; 43% yield) was purified by HPLC on a C_4 column (Vydac, Hesperia, CA), using a 0.1% TFA in H_2O/MeCN gradient.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (1H, dd, J = 11 Hz, 15 Hz, 11-H), 6.71 (1H, br s, NH), 6.29−6.14 (4H, m, H-7, H-12, H-8, and H-10), 5.75 (1H, s, H-14), 4.67 (1H, m, H-a), 4.17 (1H, d, J = 9 Hz, −CH_2−OH), 3.82 (1H, d, J = 9 Hz, −CH_2−OH), 2.36 (3H, s, H-20), 2.06 (2H, m, H-4), 1.99 (3H, s, H-19), 1.74 (3H, s, H-18), 1.61 (2H, m, H-3), 1.48 (2H, m, H-2), 1.03 (6H, s, H-16 and H-17); HRFABMS *m/z* M^+ 387.2413 (calcd for $C_{21}H_{33}0_4N$, 387.2409).

Synthesis of *N-trans*-Retinoylalanine (2) (Figure 4). RA (**3**) (20 mg, 0.066 mmol), EDC-HCl (37.8 mg, 0.198 mmol), and the free base of L-alanine methyl ester (6.8 mg, 0.066 mmol) were dissolved in 3 mL of dry CH_2Cl_2 and stirred for 20 min at room temperature and then treated with DMAP (1.6 mg, 0.0132 mmol). The reaction was stirred for 4 h and then quenched with saturated aqueous NH_4Cl. The organic layer was washed once with saturated aqueous $NaHCO_3$ and once with H_2O and then dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography (SiO_2, 4:1 hexanes−EtOAc). The isolated *N-trans*-retinoylalanine methyl ester (**11**) was redissolved in 4 mL of absolute EtOH and treated with 50 mg of KOH for 3 h. The mixture was diluted with H_2O, the aqueous solution was acidified to pH 3 with 6 N HCl, and the precipitate was extracted with ether (3 × 10 mL). The ether layer was washed with brine and then dried over Na_2SO_4. The solvent was removed in vacuo, and the yellow solid was purified by flash column chromatography (SiO_2, 10:1 $CHCl_3$−MeOH) to give 10.3 mg of *N-trans*-retinoylalanine (**2**) (42% yield) as yellow crystals: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (1H, dd, J = 11, 15 Hz, H-11), 6.32 (1H, m, H-7), 6.25 (1H, m, H-12), 6.18 (3H, br m, H-8, H-10, NH), 5.78 (1H, s, H-14), 4.66 (1H, m, alanine Cα-H), 2.42 (3H, s, H-20), 2.10 (2H, m, H-4), 2.08 (3H, s, H-19), 1.78 (3H, s, H-18), 1.69 (2H, m, H-3), 1.55 (5H, m, H-2 and alanine CH_3), 1.11 (6H, s, H-16 and H-17); HRFABMS *m/z* M^+ 371.2465 (calcd for $C_{23}H_{33}O_3N$, 371.2461).

Acknowledgment. This work was supported by grants from the National Institutes of Health (CA49933, CA89362, CA08748, and GM36564). We are also grateful for an NIH Vision Training grant, EY13933-3 (to N.F.).

References and Notes

(1) Wald, G. *Science* **1968**, *162*, 230−232.
(2) Maden, M. *Acta Biotheoretica* **1993**, *41*, 425−445.
(3) Evans, R. M.; Hollenberg, S. M. *Cell* **1988**, *52*, 1−3.
(4) Petkovitch, M.; Brand, N. J.; Krust, A.; Chambon, P. *Nature* **1987**, *330*, 444−450.
(5) Blaner, W. S.; Olson, J. A. In *The Retinoids: Biology, Chemistry and Medicine*, 2nd ed.; Sporn, M. B., Roberts, A. B., Goodman, D. W., Eds.; Raven Press: Ltd: New York, 1994; pp 209−286.
(6) Buck, J.; Derguini, F.; Levi, E.; Nakanishi, K.; Hammerling, U. *Science* **1991**, *254*, 1654−1656.
(7) Derguini, F.; Nakanishi, K.; Hammerling, U.; Chua, R.; Eppinger, T.; Levi, E.; Buck, J. *J. Biol. Chem.* **1995**, *270*, 18875−18880.
(8) Buck, J.; Grun, F.; Derguini, F.; Chen, Y.; Kimura, S.; Noy, N.; Hammerling, U. *J. Exp. Med.* **1993**, *178*, 675−680.
(9) Grun, F.; Noy, N.; Hammerling, U.; Buck, J. *J. Biol. Chem.* **1996**, *271*, 16135−16138.
(10) Hoyos, B.; Imam, A.; Chua, R.; Swenson, C.; Tong, G. C.; Levi, E.; Noy, N.; Hammerling, U. *J. Exp. Med.* **2000**, *192*, 835−846.
(11) Imam, A.; Hoyos, B.; Swenson, C.; Levi, E.; Chua, R.; Viriya, E.; Hammerling, U. *FASEB J.* **2001**, *15*, 28−30.
(12) Breitman, T. R.; Takahashi, N. *Biochem. Soc. Trans.* **1996**, *24*, 723−727.
(13) Myhre, A. M.; Takahashi, N.; Blomhoff, R.; Breitman, T. R.; Norum, K. R. *J. Lipid Res.* **1996**, *37*, 1971−1977.
(14) Shealy, F. Y.; Frye, J. L.; Schiff, L. J. *J. Med. Chem.* **1988**, *31*, 190−196.
(15) Wille, J. J.; Chopra, D. P. *Cancer Lett.* **1988**, *40*, 235−246.
(16) Hoyos, B.; Imam, A.; Korichneva, I.; Levi, E.; Chua, R.; Hammerling, U. *J. Biol. Chem.* **2002**, *277*, 23949−23957.
(17) McClean, S. W.; Ruddel, M. E.; Gross, E. G.; DeGiovanni, J. J.; Peck, G. L. *Clin. Chem.* **1982**, *28*, 693−696.

NP0496791